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Multi-location field trial of crude oil and oily sludge degrading bacterial strains at natural crude oil and oily sludge contaminated sites.

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Acknowledgement

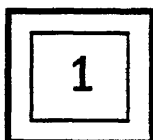
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Introduction

Aquatic and terrestrial crude oil spill incidents have been increasing at an alarming rate, despite the best efforts of the petroleum industry and consumers (reviewed by Prince 1993, Swannell et al, 1996). These spills receive much attention and evoke considerable public concern due to carcinogenicity of hydrocarbon pollutants. Among many hydrocarbon pollutants oily sludge and crude oil stand out as the major culprits, which are produced by petroleum industries, especially oil refineries (Dibble and Bartha, Mishra et al, 1979). Petroleum refining unavoidably generates huge volume of oily sludge and crude oil waste and it constitutes a disposal problem. Currently oily sludge is being disposed in open field in the refinery area and with continuous disposal of oily sludge in open field, there is a serious threat of groundwater contamination leading to various health hazards. Soil near crude oil storage tank also gets contaminated either by occasional minor spill or by pipeline leakage. Being an inflammable fluid, such spills increase the risk of fire hazards in refineries. Therefore oil refineries are suitable realistic sites for field trial of *in situ* and on site bioremediation of crude oil or oily sludge contaminated site.

There is growing public concern as a wide variety of toxic organic chemicals are being introduced into the environment. Petroleum hydrocarbons are one common example of these chemicals, which enter the environment frequently and in large volumes through numerous routes. The process of petroleum refining generates huge volumes of oily sludge whose safe disposal is a matter of great concern to all. Storage and handling of crude oil and the present disposal of the generated oily sludge onto open fields leads to soil contamination posing serious threats of groundwater contamination and increasing fire hazards at spill sites.

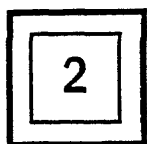
Though a wide variety of physical, chemical and biological technologies are currently being developed for the decontamination of polluted sites, in-situ bioremediation is regarded as environmental friendly because it requires little energy input, preserves the soil structure and can lead to the detoxification of contaminants.

Crude oil and its derivatives as a class represent an essential part of industrialised world. Crude oil is transported by ship, rail and truck to various

points, where it is stored in either underground or aboveground tanks in preparation for final use. During each of these processes, numerous opportunities exist for the introduction of these products into the environment. In addition to releases during the transport and storage of hydrocarbons, opportunities for mishandling and spillage exist (in addition to pilferage and terrorist activities) during the various manufacturing processes that use these products. Oily sludge generated after petroleum refining is often disposed of by methods, which introduce harmful hydrocarbons in the environment.

Bioremediation offers to restore the environment using biological means which are eco-friendly and cost-effective.

The aim of the proposed work was to conduct multilocation field trial of Oilzapper. Oilzapper is a bacterial consortium, developed with the assembly of five bacterial strains, which could degrade crude oil and oily sludge. Oilzapper was produced in bulk and immobilised on a carrier material. Carrier based Oilzapper was used as inoculum for clean-up of crude oil spill and treatment of oily sludge. Field trials of Oilzapper for clean-up of crude oil spill and treatment of oily sludge has been conducted at IOCL, Mathura refinery, Barauni refinery, Digboi refinery, BPCL refinery(Mumbai), HPCL refinery (Visakhapatnam) and Oil India Ltd(Duliajan)



Field trial of oilzapper at IOCL, Mathura refinery

Methods

Oilzapper

Crude oil/oily sludge degrading consortium has been developed with the assembly of five bacterial strains. The developed bacterial consortium could degrade crude oil/oily sludge (a hazardous hydrocarbon waste generated by oil industry) at very fast rates under laboratory and field conditions. This bacterial consortium was produced in bulk and immobilised on to a carrier material and carrier based bacterial consortium designated as Oilzapper. Bioremediation of crude oil and oily sludge contaminated sites using Oilzapper has been tested at various oil refineries and oil well sites.

Mass scale production of Oilzapper

Oilzapper (oily sludge degrading bacterial system) was produced in the fermenter (Bioflow 3000 NBS, USA) on minimal salt medium with molasses as low cost carbon and energy source. After 24 hours of growth cultures was harvested and mixed with a reusable polybags (Figure 2.1). Oilzapper packets were kept in dark at 25°C for 7 days for curing. Oilzapper packets were transported to Mathura Refinery and applied at the rate of 1kg oilzapper/10 m² area.

Selection of site and experimental design

More than 2.5 acre oily sludge contaminated land for *in situ* and on site bioremediation was selected in Mathura Refinery near scrap yard area (Figure 2.2). The total 10000 m² land area was marked off in two experiment plots. Oily sludge was heterogeneously distributed in experimental site, therefore, oily sludge was mixed in soil with a tractor fitted with harrow. After mixing of oily sludge in soil, the levels of contamination of oily sludge (solvent extractable petroleum hydrocarbon) in soil at zero time (just before experiment was initiated) was observed.



Figure 2.1 Oilzapper - a carrier based oily sludge degrading bacterial system

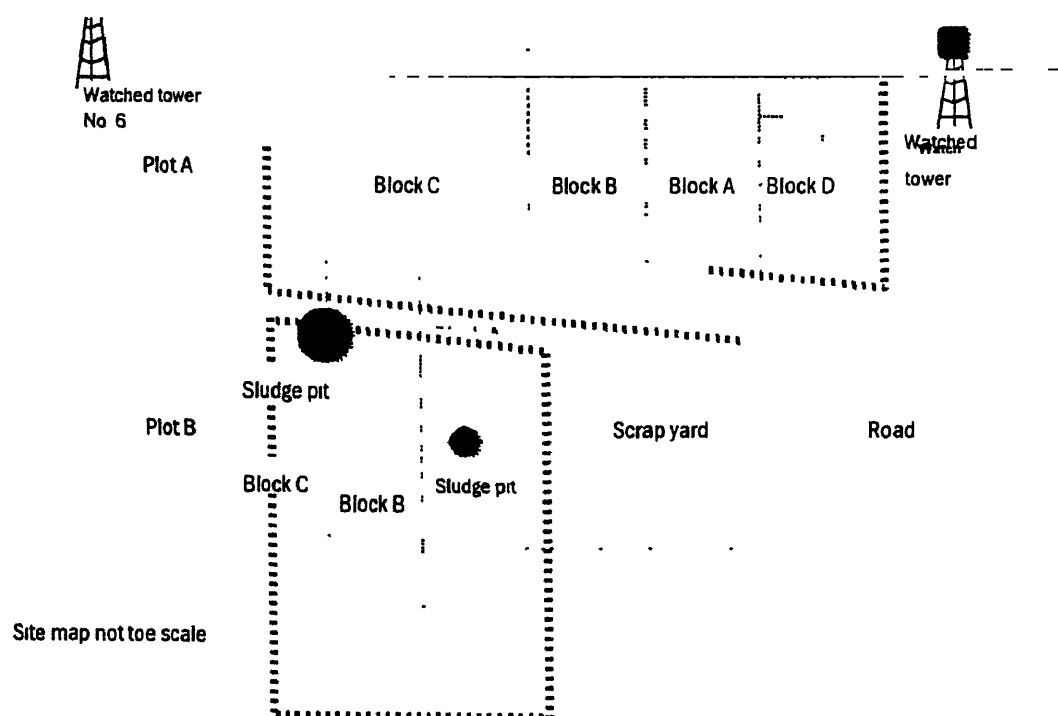


Figure 2.2 Layout of bioremediation site (Area 2.5 acre land)

Each plot was divided in three blocks and each block was sampled at 12 points to a depth of 25 cm before the experiment was initiated. Oily sludge was heterogeneously distributed in the soil samples collected from experimental blocks and therefore, the soil was powdered by soil pulverizer, mixed uniformly by passing through the sieve to uniform distribution of sludge and then solvent extractable total petroleum hydrocarbons (TPH) from 10 g soil was extracted with hexane, methylene chloride and chloroform (100 ml each) consecutively (93-95% extraction efficiency). After extraction all three extracts (obtained by hexane, methylene chloride and chloroform) were pooled and dried at room temperature by evaporation of solvents under gentle nitrogen stream in fume hood. After solvent evaporation, the amount of TPH recovered from 10 g soil collected at zero time was determined by simple gravimetry. Soil samples collected at zero time were also analysed for organic carbon, nitrogen, available phosphorus, potassium, soil, moisture, soil bulk density, soil texture, and pH.

Chromatography analysis of total petroleum hydrocarbon

TPH recovered from the sludge contaminated soil at zero time and after different time intervals was fractionated into alkane, aromatic asphaltene and NSO (nitrogen, sulphur, and oxygen containing compound) with a silica gel column. The TPH was first dissolved in n-pentane and separated into soluble and insoluble fractions (asphaltene). The soluble fraction was loaded on silica gel column and eluted with different solvents. The alkane fraction was eluted with hexane, followed by the aromatic fraction, which was eluted with benzene. The NSO fraction was eluted with methanol and chloroform. Alkane and aromatic fractions were concentrated by evaporation of solvents and then 0.2 µl of each was analysed by gas chromatography (GC Hewlett Packard, 5890 Series II) to identify all the compounds present in the alkane and aromatic fractions. In the alkane fraction, various alkane compounds were identified by matching the retention time with authentic standards. Simultaneously, the identification of each alkane compound was also confirmed by spiking the alkane fraction. A known amount of each standard alkane compound was added to the alkane fraction and injected into GC (co-injection) and the peak area of each alkane compound increased as a result. Similarly, the polycyclic aromatic hydrocarbon compounds were identified by matching the retention time with an authentic standard and confirmed by spiking the sample with authentic standards.

Simultaneously, each alkane and aromatic compound was quantified using a regression factor for each compound, drawn from a standard curve of each compound of alkane and aromatics constructed by GC.

Application of Oilzapper at Field trial site

Oilzapper was applied at the rate of 1kg Oilzapper/10m² area in block A, B and C of both the experimental plots at Mathura Refinery. A mixture of nutrient (Nitrogen and phosphorus) was also applied in experimental blocks A, B and C along with Oilzapper to achieve the C:N:P ratio 100:5:1 in oily sludge contaminated soil

Experimental plot A

Block A - treated with Oilzapper + nutrient
Block B – treated with Oilzapper + nutrient
Block C – treated with Oilzapper + nutrient
Block D – untreated control

Experimental plot B

Block A – treated with Oilzapper + nutrient
Block B – treated with Oilzapper + nutrient
Block C – treated with Oilzapper + nutrient
Block D – untreated control (Same for plot A and plot B)

After applying the Oilzapper and nutrients in experimental blocks soil was again tilled with a tractor for uniform distribution of bacterial cells. The control block D was sprayed only with water.

Quantification of oily sludge degradation

The soil of both the experimental plots was sampled at the intervals of 45 days. Soil was also tilled with a tractor at periodical intervals. Water was sprayed in both the experimental plots in summer months to maintain the moisture levels for enhance biodegrading capability of Oilzapper. Soil samples collected at periodical intervals were transported to the laboratory at Tata Energy Research Institute (TERI), New Delhi and soil was powdered by soil pulverizer, mixed uniformly by passing through the sieve to ensure uniform distribution of sludge

and then oily sludge from 10g soil was extracted with hexane, methylene chloride and chloroform (100 ml each) consecutively.

The extraction of solvent extractable total petroleum hydrocarbons (TPH) from soil were identical to that described earlier, and the amount of TPH recovered from 10g soil was quantified by simple gravimetry. After gravimetry quantification of TPH in soil at different time intervals, TPH was fractionated into various fractions on silica gel column. Alkane and aromatic fractions of TPH was analysed by gas chromatograph (GC) and quantified the amount of various alkane and aromatic compounds present in TPH at different time intervals.

Monitor the seepage of oily sludge in soil

Seepage of total petroleum hydrocarbons in soil of experimental plots was monitored by taking soil samples from different depths. At zero time (just before the initiation of experiment) and at the intervals of 45 days the plots were sampled at 25cm, 50cm, 75cm and 100cm depths.

Soil collected from 25cm, 50cm, 75cm, and 100cm depths was powdered by soil pulverizer and then total petroleum hydrocarbon (TPH) from 10g soil was extracted with hexane, methylene chloride and chloroform (100ml each) consecutively. After extraction all three extracts were pooled and dried at room temperature by evaporation of solvents under gentle nitrogen stream in fume hood. After solvent evaporation the amount of oily sludge (TPH) recovered from 10g soil was determined.

Survivability of Oilzapper at experimental site

A. baumannii strains (S19 and S30 strains which were constituent of Oilzapper) were selected to monitor the survival of the introduced bacterial strains at bioremediation site at Mathura refinery. Both the strains of *A. baumannii* were resistant to different antibiotics and can be easily distinguished from other constituent bacterial strains in the consortium by colony morphology.

Sample Collection from bioremediation site at Mathura Refinery

To monitor the survival of introduced bacterial strains at Mathura refinery soil samples were collected at regular time period from plot A and B. Soil samples were also collected at the end of study from these plots. Similarly soil samples were also collected from plot C at time zero and after completion of study. Soil samples (1 g) from all the three plots were suspended in saline water (0.85%

NaCl). The suspensions, after appropriate dilution were plated onto Luria Bertani Agar (LA) containing spectinomycin and ampicilin (50 µg/ml each) in one set of petriplates to estimate the population of *A. baumannii* S19. Similarly the suspension was plated onto LA plates containing 20 µg/ml vancomycin and 50 µg/ml of ampicilin to estimate the population of *A. baumannii* S30. Bacterial colonies (100 colonies) from soil samples of plot A and B were picked randomly from selective LA plates of 10⁵ dilution. Bacterial colonies did not grow from the soil samples of plot C when plated onto selective LA plates (one set of LA plate containing 20 µg/ml vancomycin, 50 µg/ml ampicilin and other set of LA plate containing spectinomycin and ampicilin 50 µg/ml each). These colonies were subjected to Enterobacteriaceae Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) to obtain DNA fingerprints. At time zero the number of colonies picked for ERIC-PCR represented more than 58% of total number of colonies and after one year it represented more than 82% for the total number of colonies. The primers ERIC-1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC-2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3'), were obtained from Life Technologies, USA. Single isolated colonies were picked at random from the selective LA plates, suspended in 50 µl water and lysed by heating for 10 min at 95 °C. The cell lysate was centrifuged at 12000 revolution per minute (rpm) and 4 °C for 5 minute and 2 µl of the supernatant was used in the reaction mixture. The reaction mixture (15µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.01% gelatine (w/v), 0.2 mM (each) of dNTPs, 1µM each of primers ERIC-1R and ERIC-2 and 0.45 U of Taq polymerase (Life Technologies, USA). The mixture was overlaid with 20 µl mineral oil and amplification was done on DNA engine PTC-200 (MJ Research, USA). The amplification protocol included initial denaturation at 95 °C for 2 min followed by 35 cycles at 92 °C for 30 sec, 50 °C for 1 min.20 sec and 68 °C for 3 min. 20 sec. The final extension was done at 68 °C for 8 min. The reaction was terminated using loading dye (1µl) containing 15% Ficoll, 0.25% bromophenol blue and 0.25% xylene cyanol. Each PCR product was resolved by electrophoresis on a 2% agarose gel. The fingerprint of unknown bacterial isolates thus obtained was compared with the fingerprint of *A. baumannii* strains (S19 and S30) obtained under similar reaction conditions to confirm the survival of the strains at the contaminated site.

Survival of Lux-gene marked A.baumannii in oil contaminated soil

Acinetobacter baumannii S30 and S19 pJES containing Lux gene was grown in basal medium for 48 hrs. The final CFU of *A. baumannii* S30 was 7×10^8 / ml and that of *A. baumannii* S19 was 1.3×10^8 / ml. The cells were centrifuged and suspended in 20 ml of sterile basal medium. In a sterile glass plate, 100 g of contaminated soil was taken. One of the plates was kept as control where no bacterial culture was added. In the other two plates 10 ml cultures of *A. baumannii* S30 and S19 pJES were added and mixed thoroughly so that a final CFU 2×10^9 /g of soil for S30 pJES and 2.6×10^9 /g of soil for S19 pJES is maintained. At regular interval, soil samples were collected, dilution plated onto antibiotic and non-antibiotic plates. Total DNA from each plate was also collected using Total DNA isolation kit from Mo Bio, USA. DNA obtained was subjected to LUX-PCR

Analysis of trace metals in soil at bioremediation site

Various trace metals were analysed from soil samples collected at zero time and after one year from experimental site. Soil samples were digested in nitric acid. A known weight of soil was taken in clean 100 ml beaker. A 15 ml concentrated nitric acid was added to beaker and the beaker was covered with a watch glass and heated at 140°C on a hot plate, in a fume hood, until most of the acid was evaporated. The step was repeated thrice in order to dissolve the metallic components. The solution was then filtered in another beaker through 0.45 micron Whatman filter paper number 42 and the insoluble residues on the filter paper was rinsed with 10% nitric acid. The residue was then discarded and beaker was covered with a watch glass and heated at 140°C until complete evaporation of nitric acid. Now the beaker was heated at 400°C until barely dried and white ash appeared. The sample was not allowed to bake and temperature was maintained at 400°C for six hours. The bottom of the watch glass was carefully rinsed into the beaker using 10% nitric acid. The sides of the beaker was also rinsed and solution was evaporated to dryness at 140°C . The filter paper blank and nitric acid blank were also prepared similarly. Each beaker was then cooled and the residue was dissolved in 1 ml of concentrated nitric acid. The clear solution was then quantitatively transferred into 50 ml volumetric flask and volume was made up by using 10% nitric acid. The extract was initially analysed by flame (air-acetylene) AAS using standard method. Some metals such as Se, and As, were analysed by AAS equipped with hydrate

system. Metals were also analysed in soil samples collected at different depths of experimental plot.

Analysis of trace metals in oily sludge biodegraded at Mathura Refinery

A known weight of sludge was taken in a clean beaker. Concentrated nitric acid (15 ml) was added to the beaker and the beaker was covered with a watch glass and heated at 140°C on a hot plate, in a fume hood, until most of acid was evaporated, the step was repeated five times in order to dissolve the metallic components. The solution was then filtered in another clean beaker through 0.45 µ Whatman filter paper number 42 and the insoluble residues on the filter paper was rinsed with 10% nitric acid. The residue was then discarded. The beaker containing filtered solution was again heated till complete evaporation of acid. After evaporation of acid beaker was heated at 400°C for six hours. Beaker was then cooled and the residue was dissolved in 1 ml of concentrated nitric acid. The clear solution was then quantitatively transferred into 50 ml volumetric flask and volume was made by using 10% nitric acid. The trace metals from solution was analysed using AAS (Atomic absorption spectrophotometer) as described earlier.

Results

Soil analysis of bioremediation site

Before initiation of bioremediation experiment (zero time) and after completion of experiment (after 360 days) the soil (up to 25 cm) was collected from the experimental site. At zero time the soil was completely black due to oily sludge contamination. No vegetation was existed except few grasses. After six months of initiation of bioremediation experiment the site was full with vegetation. Soil analysis of experimental site showed 3.2% and 2.6% organic carbon at zero time and after completion of experiment (after 360 days) respectively. Available phosphorus in soil of experiment site increased from 12 ppm to 27 ppm after 360 days. However the potassium concentration in soil was not changed significantly during the experiment (Table 2.1). The total nitrogen in soil increased from 0.046% to 1.12%, whereas the soil pH and soil bulk density of experimental site was not changed significantly from zero time to completion of experiment (Table 2.1). Soil texture and water holding capacity of soil was also changed during experiment (Table 2.1).

Table 2.1 Physical and chemical properties of soil of experimental site at Mathura Refinery

Test	Results	
	Zero time	After 360 Days
Physical Properties		
Texture	Black loam	Loam
Bulk density	1.3gm/cm ³	1.35 g/cm ³
Water holding capacity	59%	71%
Chemical properties of soil		
PH	7.4	7.6
Organic carbon	3.2%	2.6%
Total nitrogen	0.046%	1.15%
Potassium	130ppm	115ppm
Available phosphorus	12ppm	27ppm

Composition of oily sludge

Oily sludge biodegraded at Mathura Refining contained 18% solvent extractable total petroleum hydrocarbon (steam extractable total TPH 4%), organic carbon 41%, sediment/ash content 35% and water 6%. Solvent extractable total petroleum hydrocarbons extracted at zero time from contaminated soil was fractionated into various fractions showed 52% alkane, 31% aromatic, 10% asphaltane and 7% NSO.

In-situ bioremediation of oily sludge contaminated soil at Mathura Refinery

Two experimental plots (Plot A and Plot B) with total area 2.5 acre land contaminated with oily sludge was selected for bioremediation. In experimental plot A, the initial concentration of oily sludge (Solvent extractable TPH) in soil of block A was 13.41%. With application of Oilzapper plus nutrient the oily sludge concentration (Solvent extractable TPH) in soil reduced from 13.41% to 0.50% after one year indicating a degradation of 96.2%. With application of Oilzapper plus nutrient the sludge concentration (Solvent extractable TPH) in soil of treatment block A after 45, 90, 135, 180 and 270 days was 10.40%, 5.50%, 3.25%, 2.90% and 1.40% showing a reduction of 22.4%, 58.9%, 75.7%, 78.3% and 89.5% respectively (Figure 2.3).

Similarly with application of Oilzapper and nutrient in the treatment block B of plot A, the oily sludge concentration (Solvent extractable TPH) in soil reduced from 4.36% to 0.42% after one year indicating a degradation of 90.3%. The oily sludge concentration (Solvent extractable TPH) in soil of block B after 45, 90, 135, 180 and 270 days was 2.680%, 0.90%, 0.980%, 0.50% and 0.47% revealing

a degradation of 38.5%, 79.3%, 77.5%, 88.5% and 89.2% respectively (Figure 2.3).

With application of Oilzapper plus nutrient in block C the oily sludge concentration (Solvent extractable TPH) in soil reduced from 3.19 % to 0.34 %

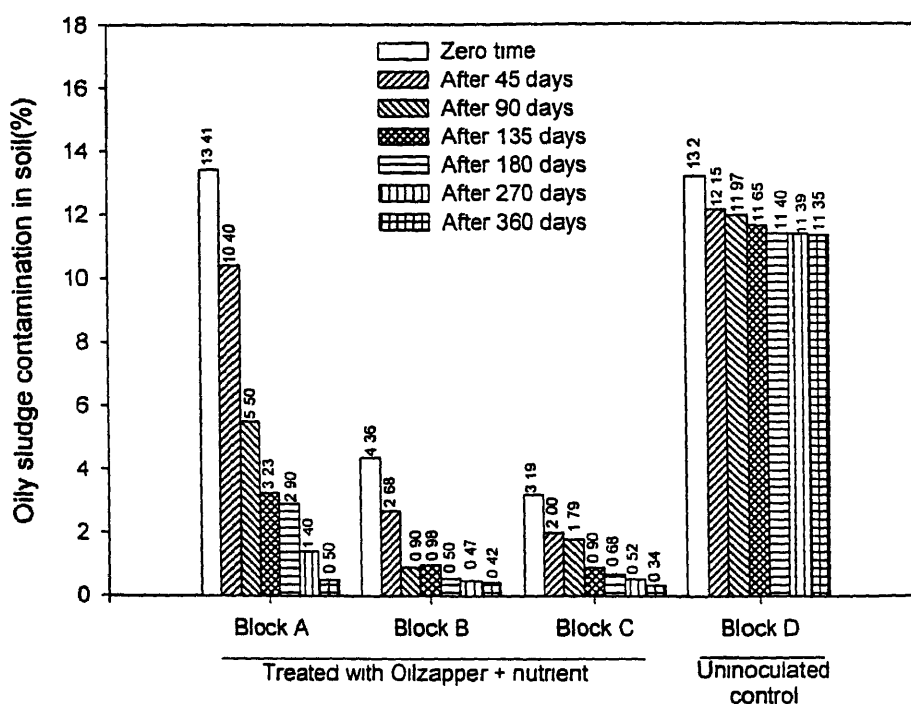


Figure 2.3 In-situ degradation of oily sludge in soil of experimental plot A

after one year showing a degradation of 89.3%. The sludge concentration after 45, 90, 135, 180, and 270 days was 2.00%, 1.79%, 0.90%, 0.68% and 0.52% indicating a degradation of 37.3%, 43.8%, 71.7%, 78.6% and 83.6% respectively (Figure 2.3).

However without application of Oilzapper the sludge concentration (Solvent extractable TPH) in soil of block D reduced from 13.20%, 11.35% after one year indicating a loss of only 14% (Figure 2.3), which may be due to various abiotic and biotic factors. The sludge concentration in soil of untreated block D after 45, 90, 135, 180 and 270 days was 12.15%, 11.97%, 11.65%, 11.40% and 11.39% showing a loss of 7.90%, 9.30%, 11.70%, 13.60% and 13.70% respectively.

In experimental plot B, the oily sludge concentration (Solvent extractable TPH) was also distributed heterogeneously and some area contained more quantity of sludge, therefore, the plot was divided into three blocks. With application of Oilzapper along with nutrient the sludge concentration in soil of block A reduced from 7.52% to 0.80% after 270 days indicating a degradation of 89.3%. The sludge concentration after 30, 75 and 210 days was 6.22%, 1.66% and 0.80% indicating a degradation of 17.2%, 77.9% and 89.3% respectively (Figure 2.4).

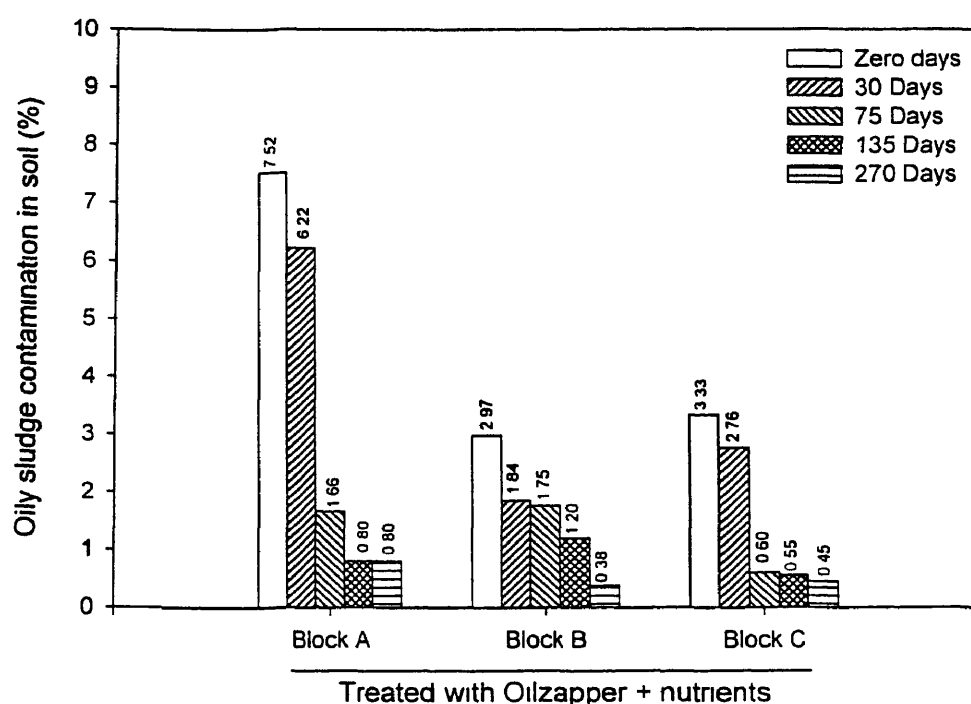


Figure 2.4 In-situ degradation of oily sludge in experimental plot B

With application of Oilzapper and nutrient in block B of plot B the oily sludge concentration (Solvent extractable TPH) was reduced from 2.97% to 0.38% after 270 days indicating a degradation of 87.2%. The sludge concentration in soil of block B after 30, 75 and 210 days was 1.84%, 1.75% and 1.20% showing a degradation of 38% 41% and 59.5% respectively (Figure 2.4).

With application of Oilzapper plus nutrient in block C of plot B the sludge concentration (Solvent extractable TPH) reduced from 3.33% to 0.45% after 270

days indicating a degradation of 86.4%. In block C the sludge concentration after 30, 75 and 210 days was 2.76%, 0.60% and 0.55% indicating a degradation of 17.1%, 81.9% and 83.4% respectively (Figure 2.4).

In-situ biodegradation of various fractions of oily sludge

Degradation of various fractions of oily sludge was quantified by simple weight loss technique. Further degradation of individual n-alkane and aromatic compounds was analysed by gas liquid chromatography. Block A of plot A treated with Oilzapper plus nutrient showed 96.2% degradation of oily sludge in 360 days, of which alkane and aromatic fractions was accounted 50.4% and 29.8% respectively (Table 2.2). GC finger printing of In-situ biodegradation of alkane and aromatic fractions of oily sludge has shown in figure 2.5 and figure 2.6 respectively. In treatment block A, the total alkane & aromatic fractions (present in oily sludge) were degraded to the extent of 97% and 96.2% respectively (Figure 2.5 and 2.6).

The total alkane and aromatic compounds were degraded to the extent of 95.80% and 91% respectively (Table 2.2).

Table 2.2 *In-situ* degradation (%) of various fractions of oily sludge in experimental plot A at Mathura Refinery

	Treatment Blocks			
	A	B	C	D
Oily sludge	96.2	90.3	89.3	14.0
Alkane fraction	50.4	49.8	46.5	9.0
	(97)	(95.8)	(91)	(17.3)
Aromatic fraction	29.8	28.2	27.4	4.0
	(96.2)	(91)	(88.4)	(12.9)

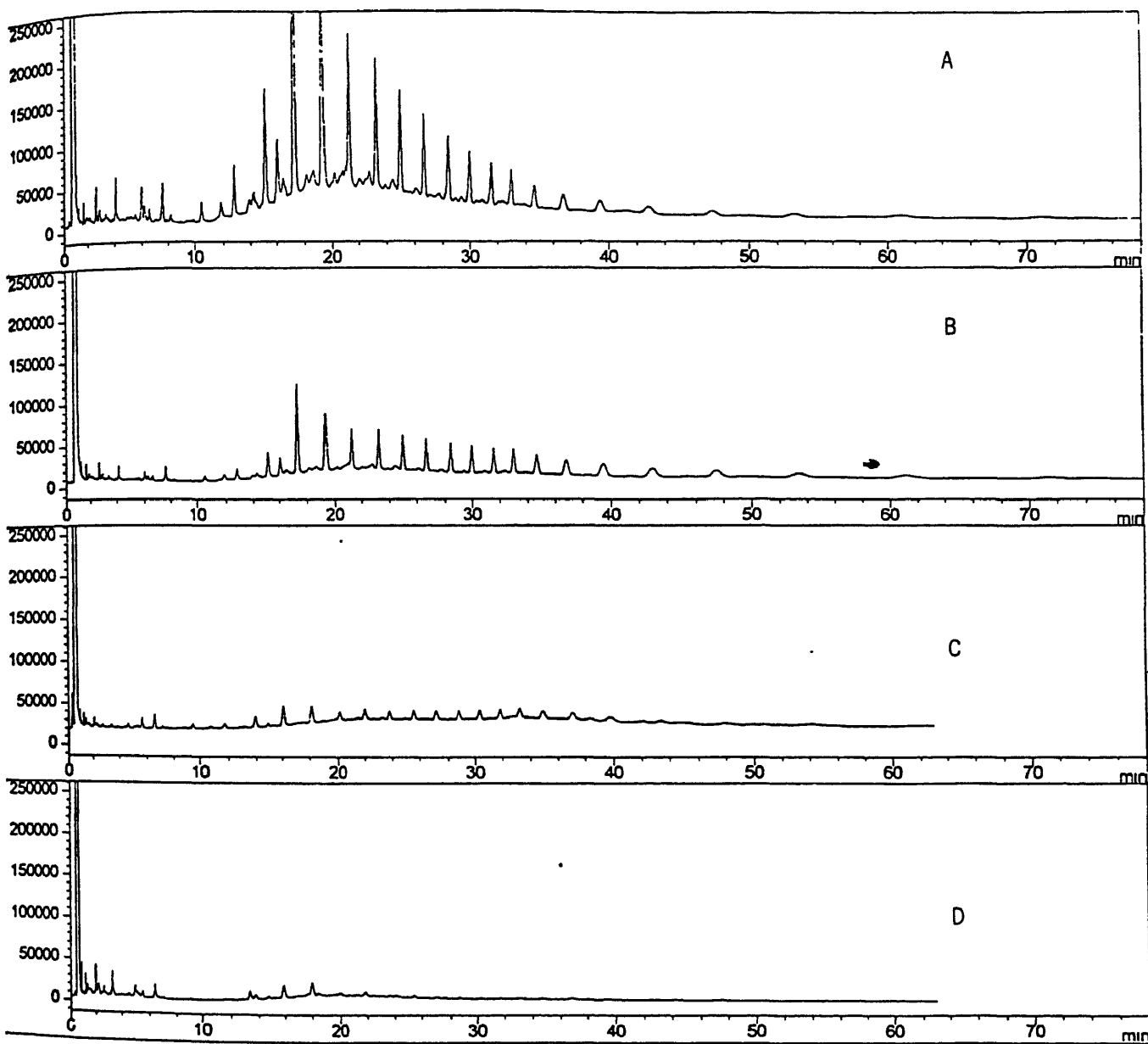


Figure 2.5 *In-situ* degradation of alkane fraction of oily sludge in experimental plot A

- A. Alkane fraction of oily sludge at zero time (just before initiation of experiment)
- B. Alkane fraction of oily sludge after 90 days of initiation of experiment
- C. Alkane fraction of oily sludge after 180 days of initiation of experiment
- D. Alkane fraction of oily sludge after 360 days of initiation of experiment

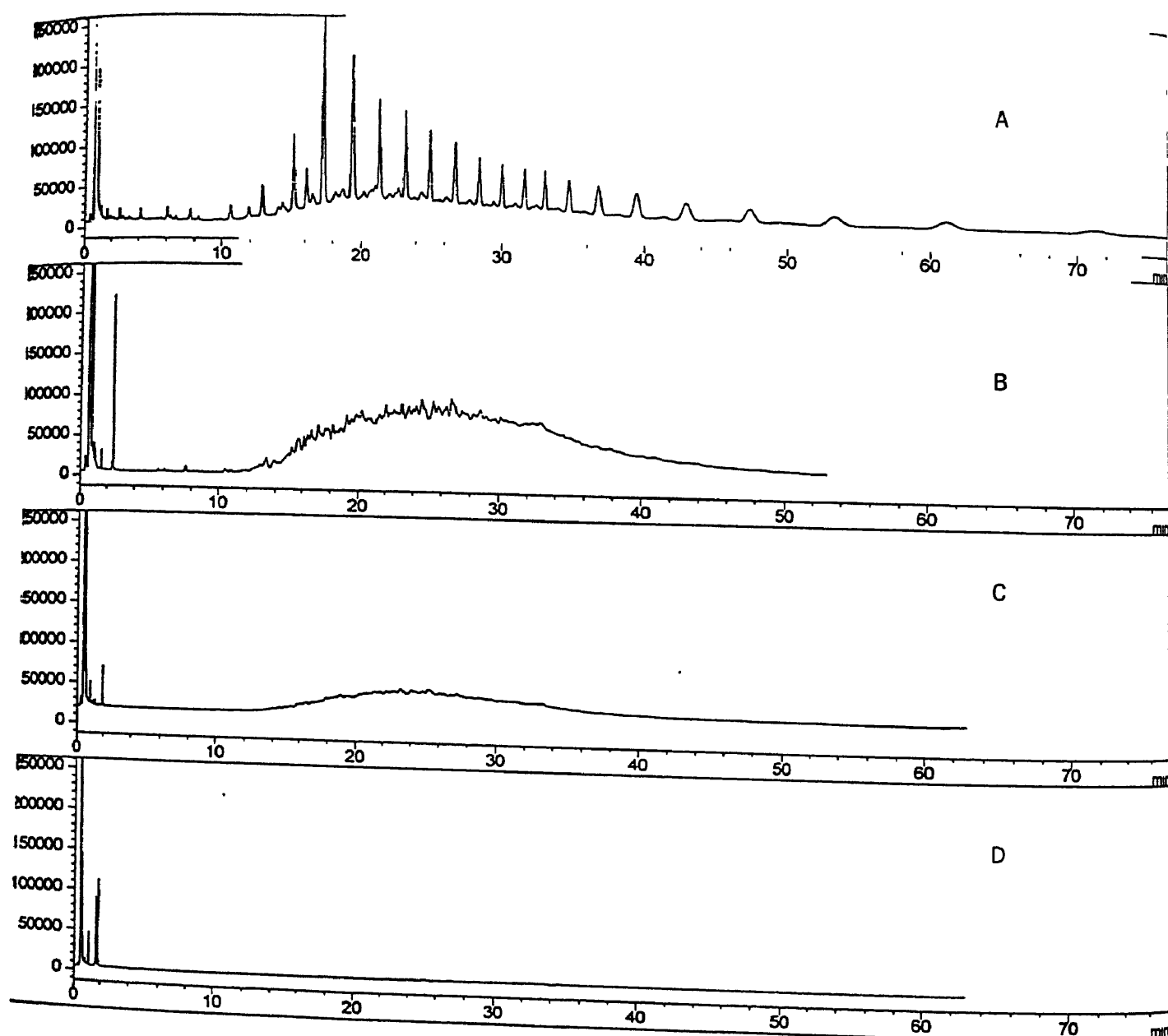


Figure 2.6 *In-situ* degradation of aromatic fraction of oily sludge in experimental plot A

- A. Aromatic fraction of oily sludge at zero time (just before initiation of experiment)
- B. Aromatic fraction of oily sludge after 90 days of initiation of experiment
- C. Aromatic fraction of oily sludge after 180 days of initiation of experiment
- D. Aromatic fraction of oily sludge after 360 days of initiation of experiment

Among the lkane fraction of oily sludge long chain alkane compounds were degraded extensively, which are normally difficult to degrade in contaminated soil. In long chain alkane the major degradation was of hexadecane ($C_{16}H_{34}$), heptadecane ($C_{17}H_{36}$), octadecane ($C_{18}H_{38}$) and eicosane ($C_{20}H_{42}$) as shown in Figure 2.7. Similarly among aromatic fraction or oily sludge, polycyclic

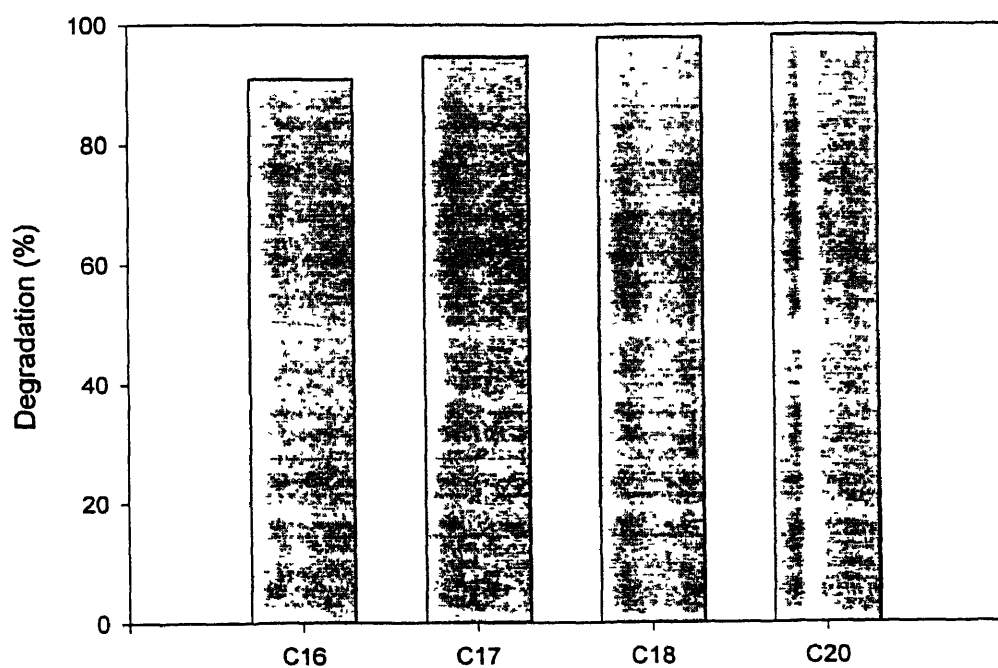


Figure 2.7 In-situ degradation of alkane compounds in plot A

aromatic hydrocarbons such as anthracene, chrysene, carbazole, and fluoranthene which are carcinogenic were degraded extensively (Figure 8).

Out of 90.30% degradation of oily sludge in treatment block B of plot A, the alkane and aromatic fractions were accounted 49.80% and 28.20% respectively.

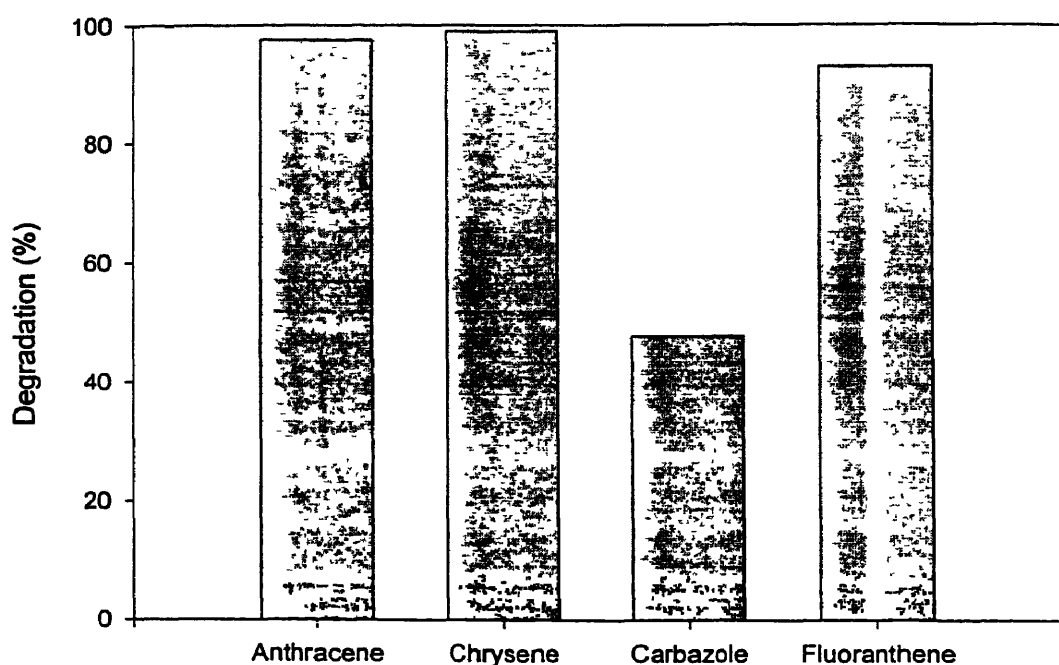


Figure 2.8 In-situ degradation of polycyclic aromatic hydrocarbon compounds in plot A

Similarly in treatment block C of plot A, out of 89.3% degradation of oily sludge, Alkane and aromatic fractions were accounted 46.5% and 27.4% respectively (Table 2.2). The total alkane and aromatic fractions were degraded to the extent of 91% and 88.4% respectively.

In untreated block D only 14% oily sludge was degraded of which alkane accounted 9% and aromatic accounted 4% (Table 2.2). The total degradation of alkane and aromatics were only 17.3% and 12.9% respectively (Figure 2.9 and 2.10).

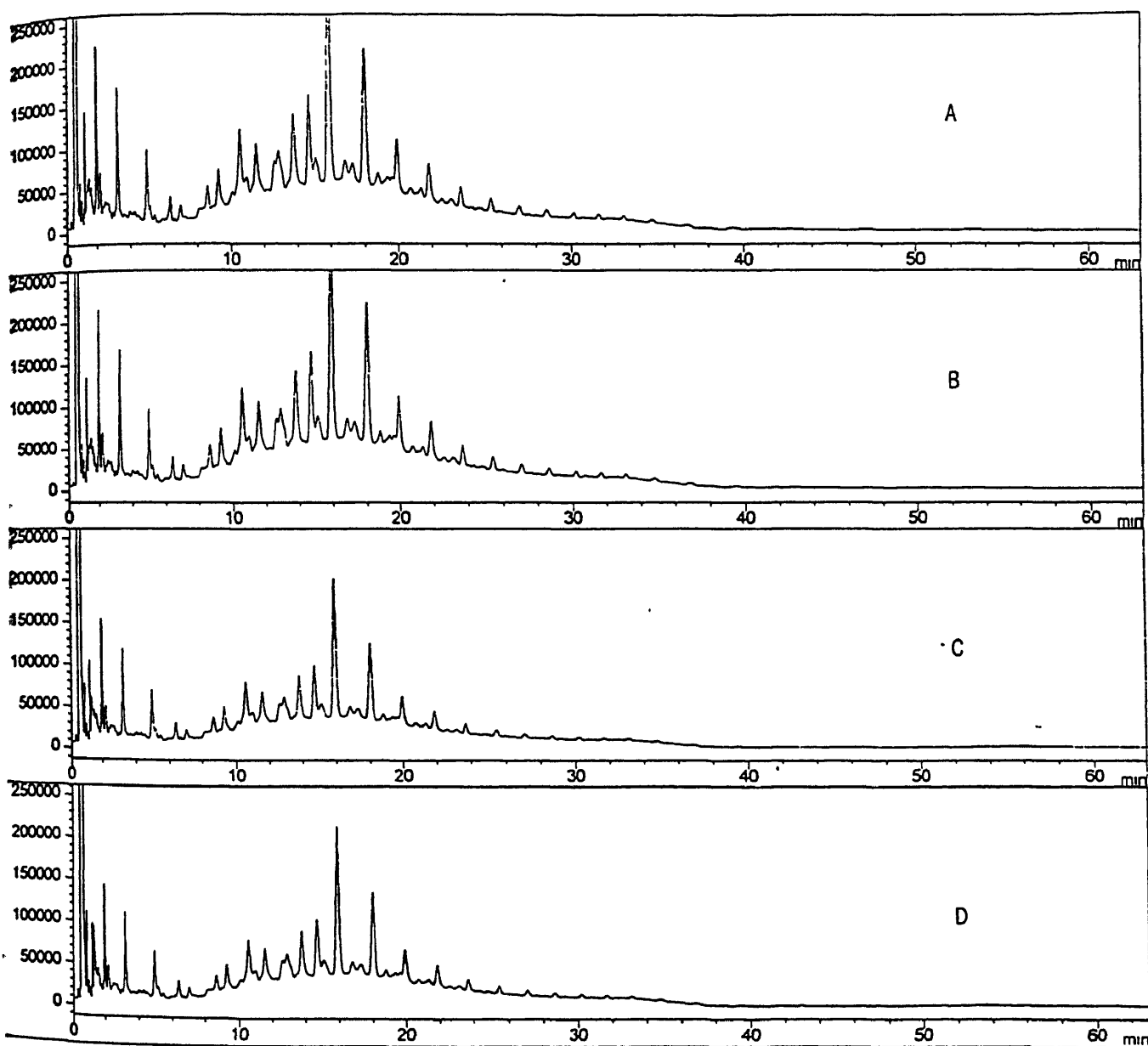


Figure 2.9 *In-situ* degradation of alkane fraction of oily sludge in untreated block D

- A. Alkane fraction of oily sludge at zero time (just before the initiation of experiment)
- B. Alkane fraction of oily sludge after 90 days of initiation of experiment
- C. Alkane fraction of oily sludge after 180 days of initiation of experiment
- D. Alkane fraction of oily sludge after 360 days of initiation of experiment

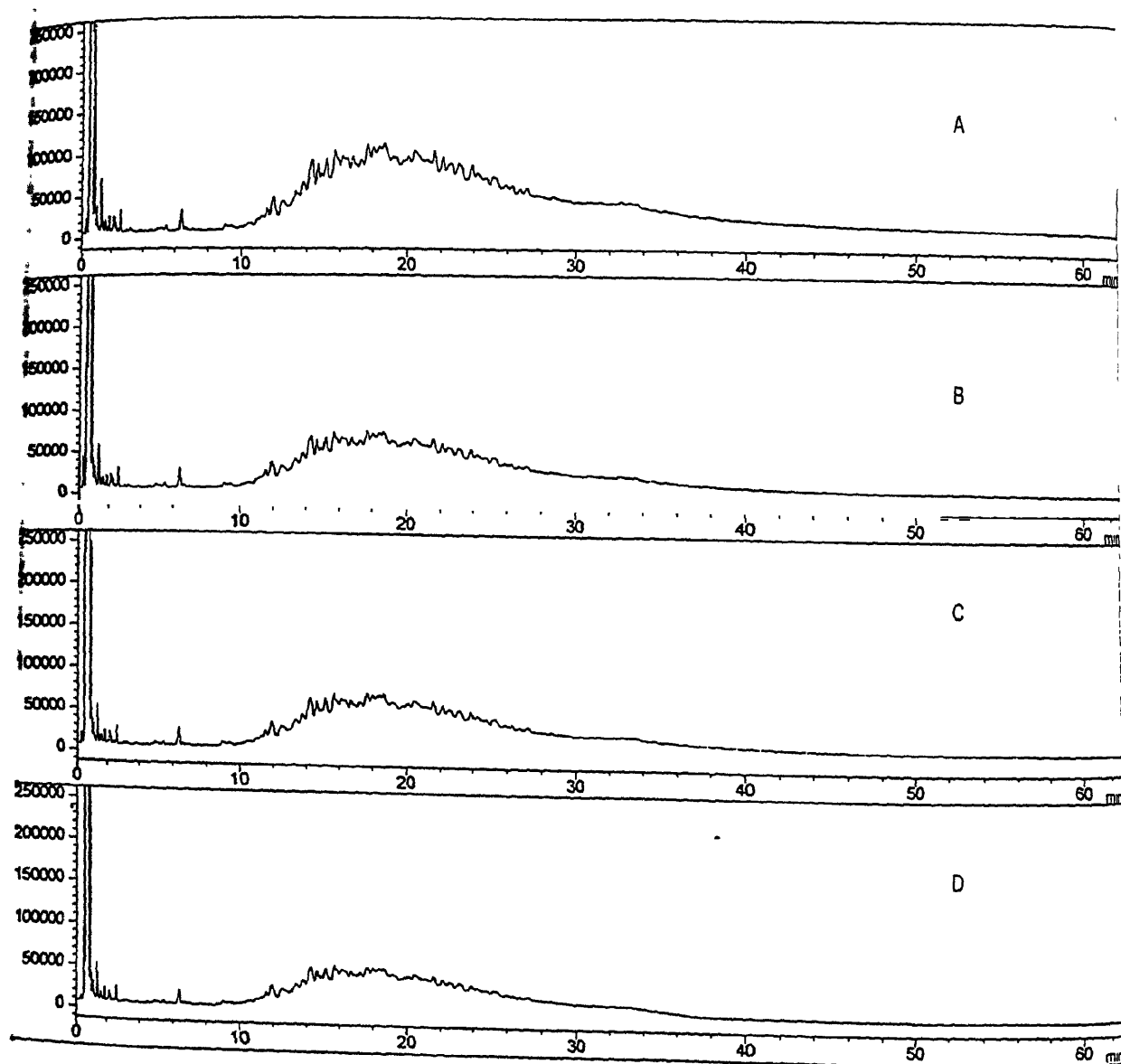


Figure 2.10 In-situ degradation of aromatic fraction of oily sludge in untreated block D

- A. Aromatic fraction of oily sludge at zero time (just before initiation of experiment)
- B. Aromatic fraction of oily sludge after 90 days of initiation of experiment
- C. Aromatic fraction of oily sludge after 180 days of initiation of experiment
- D. Aromatic fraction of oily sludge after 360 days of initiation of experiment

Block A of plot B was treated with Oilzapper plus nutrient resulted 89.30% degradation of oily sludge and out of which the degradation of alkane and aromatic fractions were 50.70% and 27.50% respectively (Table 2.3). The total alkane and aromatic compounds were degraded up to 97.50% and 88.70% respectively. Among the alkane long chain alkane compounds such as hexadecane, heptadecane, octadecane and eicosane were degraded extensively (Figure 2.11). Similarly among aromatic fraction, polycyclic aromatic hydrocarbon compounds such as anthracene, carbazole, chrysene and fluoranthene were degraded extensively (Figure 2.12).

Table 2.3 *In-situ* degradation (%) of various fractions of oily sludge in experimental plot B

	Treatment Blocks		
	A	B	C
Oily sludge	89.3	87.2	86.4
Alkane fraction	50.7 (97.5)	44.9 (86.3)	51.0 (98.0)
Aromatic fraction	27.5 (88.7)	29.0 (93.5)	30.0 (97)

Similarly in block B of plot B, the oily sludge was degraded to the extent of 87.2% of which alkane fraction was accounted 44.9% and aromatic fraction was accounted 29% (Table 2.3). In block C of plot B, the degradation of oily sludge was 86.4%. The degradation of alkane and aromatic fractions were accounted 51% and 30% respectively (Table 2.3). The total degradation of alkane and aromatic compounds was 98% and 97% respectively (Table 2.3).

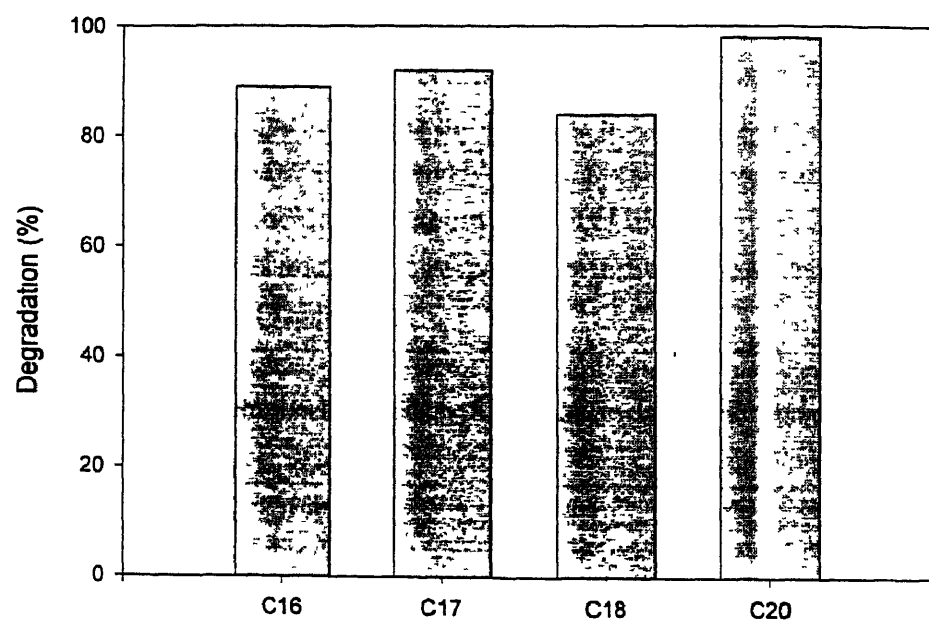


Figure 2.11 In-situ degradation of alkane compounds in plot B

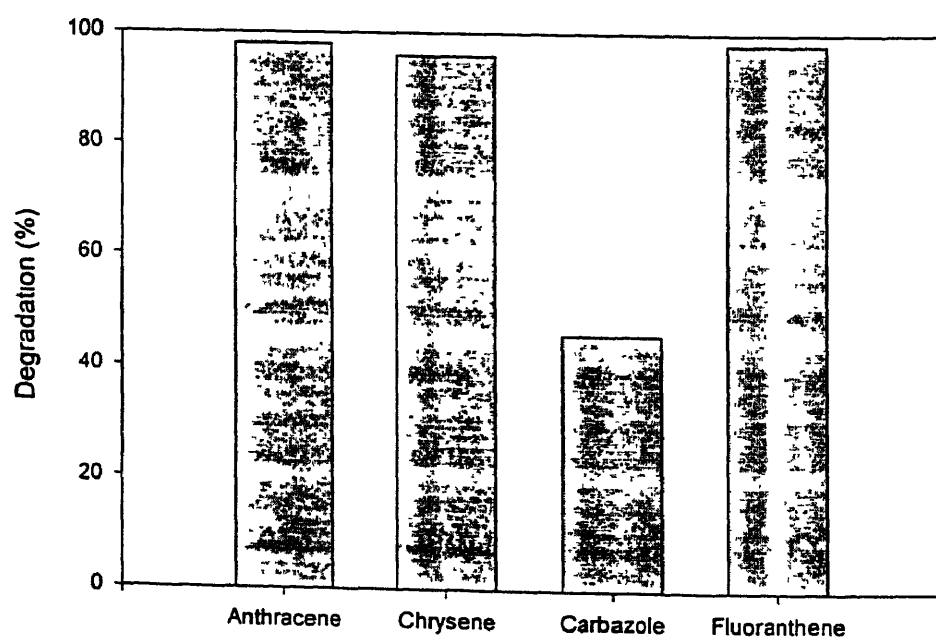


Figure 2.12 In-situ degradation of polycyclic aromatic hydrocarbon compounds in plot B

Monitor the seepage of oily sludge in soil at experimental site

Seepage of oily sludge in soil was monitored by taking soil samples from different depth. At zero time the total petroleum hydrocarbon (TPH) could not be detected below 50 cm depth of both the experimental plots. At 50 cm depth the total petroleum hydrocarbons in blocks A, B, C, and D of plot A was 0.03%, 0.03%, 0.02% and 0.02% respectively (Table 2.4). However after 360 days the total petroleum hydrocarbon at 50 cm depths of blocks A, B, C, and D was 0.02%, 0.03%, 0.01% and 0.02% respectively indicating no seepage of TPH over a period of one year (Table 2.4).

Table 2.4 Total petroleum hydrocarbon (%) at different depth of experimental plot A

Blocks	25 cm depth		50 cm depth	
	Zero time	After 360 days	Zero time	After 360 days
A	13.41	0.50	0.03	0.02
B	4.26	0.42	0.03	0.03
C	3.19	0.34	0.02	0.01
D	13.20	11.35	0.02	0.02

Note: TPH could not be detected at 75 cm and 100 cm depths

Similarly at 50 cm depth in experimental blocks A, B, and C of plot B, the TPH at zero time was 0.01%, 0.01% and 0.02% respectively and after 270 days the TPH at 50 cm depth of same blocks was 0.01%, 0.01% and 0.02% respectively revealing no seepage of oily sludge in soil (Table 2.5).

Table 2.5 Total petroleum hydrocarbon (%) at different depth of experimental plot B

Blocks	25 cm depth		50 cm depth	
	Zero time	After 360 days	Zero time	After 360 days
A	7.52	0.80	0.01	0.01
B	2.97	0.38	0.01	0.01
C	3.33	0.45	0.02	0.02
D	13.20	11.35	0.02	0.02

Note: TPH could not be detected at 75 cm and 100 cm depths

Survivability of Acinetobacter baumannii (constituent of Oilzapper) at experimental site

A high survivability of *Acinetobacter baumannii* S30 and S19 was found in Mathura refinery. The population of *A. baumannii* in plot A at the start of the

study was 1.79×10^7 CFU/g of soil which had reduced to only 1.21×10^7 CFU/g of soil at the end of 360 days. A survivability of 67.59% was noted. In plot B the population of *A. baumannii* was 1.770×10^7 CFU/g of soil at zero time which reduced to 1.01×10^7 CFU/g of soil after 360 days thus depicting a survivability of 57%. This shows that that reclamation of the contaminated land was possible because of these micro-organisms present in the consortium.

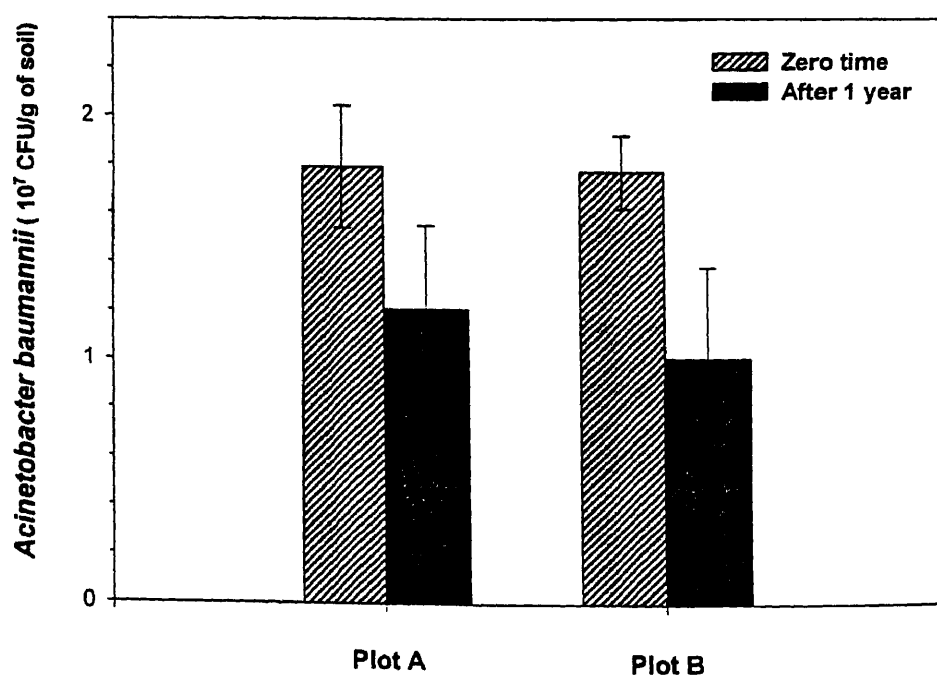


Figure 2.13 Population of *A.baumannii* in oily sludge contaminated site at Mathura refinery

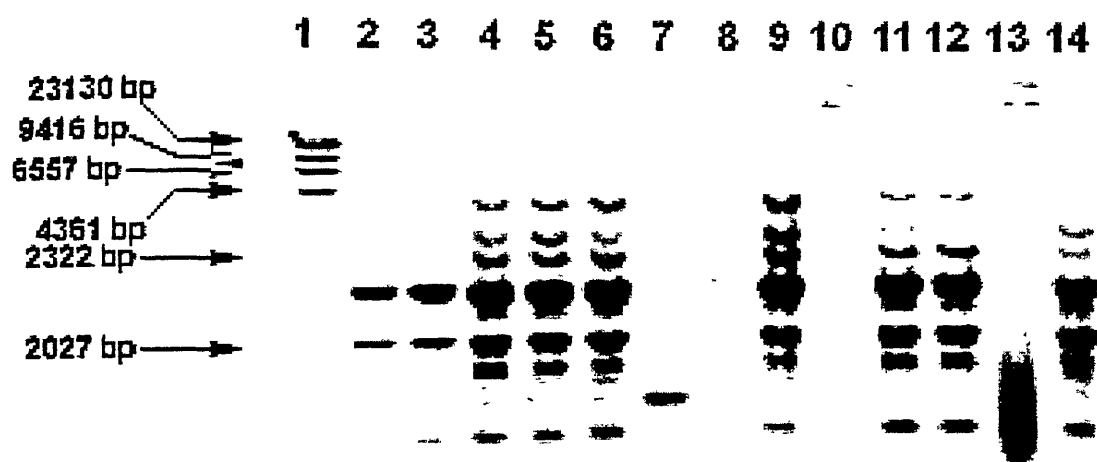


Figure 2.14 Genomic DNA fingerprints of unknown representative bacterial isolates.

Lanes 1, λ HindIII digest; 2,3,7,8,10 & 13, fingerprints of unknown isolates obtained on non-selective LA plates from plot C; 4,5 & 6, fingerprints of unknown isolates obtained on selective LA plates from plot A, 9,11 & 12 fingerprints of unknown isolates obtained on selective LA plates from plot B; 14, fingerprint of *A.baumannii* S30.

Survival of Lux-gene marked A.baumannii in crude oil contaminated soil under control conditions.

The results in (Table 2.6) depicts that the population of *Acinetobacter baumannii* S30 and S19 pJES obtained at different time interval and Figure 2.16 shows the LUX-PCR of a representative sample at zero day and after 50 days.

Table 2.6 Population of LUX gene tagged *A.baumannii* in oil contaminated soil

Time (days)	Population of S S30 pJES /g of soil	No. of colonies picked for Lux-PCR	No. +ve	Population of S-19 pJES /g of	Colonies picked - for Lux-PCR	No. Total Soil DNA +ve Lux- PCR
zero day	2×10^9	50	50	1.8×10^9	30	30 All positive
5	2.1×10^8			1.9×10^9		All positive
10	1.95×10^9			1.8×10^9		All positive
15	2×10^9	50	50	2.3×10^9	50	50 All positive
20	2.2×10^9			2.1×10^9		All positive
30	2.1×10^9	50	47	1.5×10^9	50	43 All positive
40	2×10^9			1.3×10^9		All positive
50	1.1×10^8	50	27	0.8×10^7	30	16 All positive

LUX-Polymerase Chain Reaction

During the laboratory-scale study of survivability, LUX-based PCR was conducted to confirm the presence of Lux gene. Bacterial colonies were picked randomly from selective (with antibiotic) LA plates for analysis of DNA fingerprints.

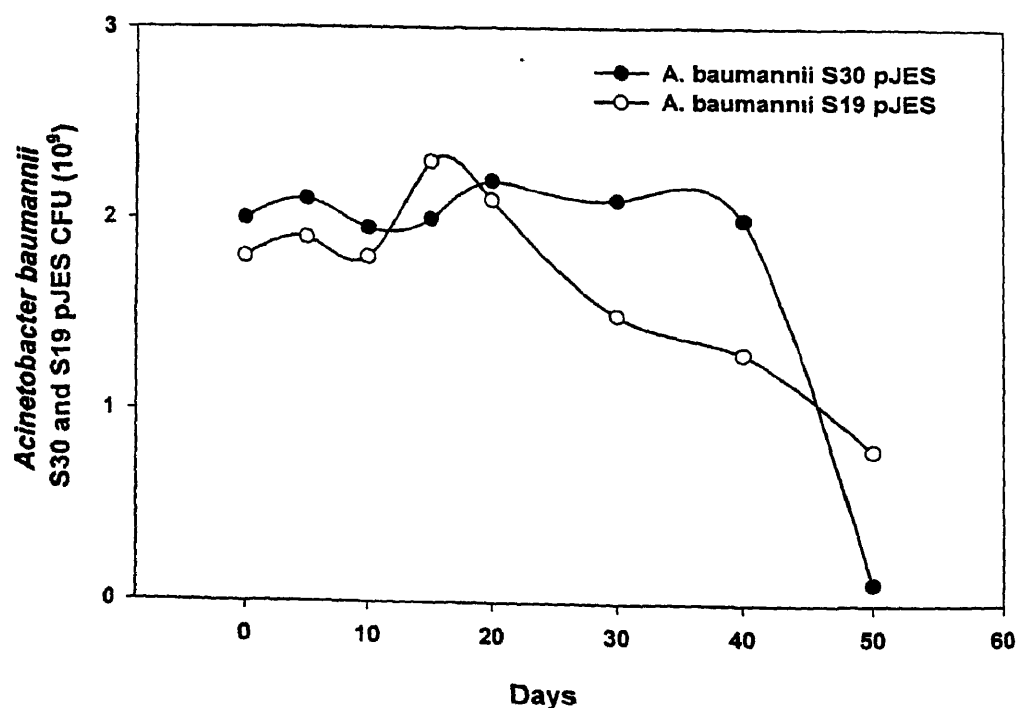


Figure 2.15 Population of LUX gene tagged *A.baumannii* in hydrocarbon contaminated soil

These colonies were subjected to LUX-Polymerase Chain Reaction to obtain DNA fingerprints. The primers used for the PCR reaction were JLCW (5'-ATG AAG TTT GGA AAT ATT TGT TTT TC-3') and JLCCW (5'-TTA TGG TAA ATT CAT TTC GAT TTT TTG-3') were obtained from Life Technologies, USA. Single isolated colonies were picked at random, suspended in 50 μ l water and lysed by heating for 10 min at 95 $^{\circ}$ C. The cell lysate was centrifuged at 12000 revolution per minute (rpm) and 4 $^{\circ}$ C for 5 minute and 2 μ l of the supernatant was used in the reaction mixture. The reaction mixture (15 μ l) contained 10 mM Tris-HCl

(pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.01% gelatine (w/v), 0.2 mM (each) of dNTPs, 0.4µM each of primers JLCW and JLCCW and 0.45 U of Taq polymerase (Life Technologies, USA). The mixture was overlaid with 20 µl mineral oil and amplification was done on DNA engine PTC-200 (MJ Research, USA). The amplification protocol included initial denaturation at 95 °C for 2 min followed by 35 cycles at 92 °C for 30 sec, 50 °C for 1 min.20 sec and 68 °C for 3 min. 20 sec. The final extension was done at 68 °C for 8 min. The reaction was terminated using loading dye (1µl) containing 15% Ficoll, 0.25% bromophenol blue and 0.25% xylene cyanol. Each PCR product was resolved by electrophoresis on a 2% agarose gel. The fingerprint of unknown bacterial isolates thus obtained was compared with the fingerprint of *A. baumannii* strains S19 pJES and S30 pJES obtained under similar reaction conditions to confirm the survival of the strains at the contaminated site. Similarly 2 µl of total DNA isolated was directly used for the PCR amplification reaction. The fingerprints obtained were again compared with the standard strains and presence of Lux gene in the soil was confirmed.

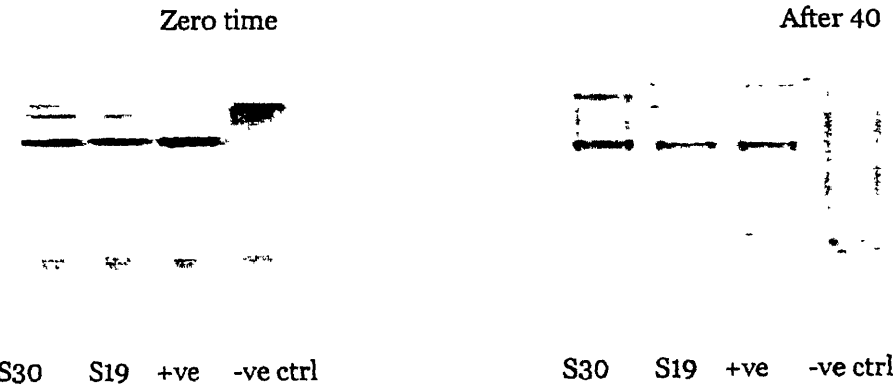


Figure 2.16 DNA fingerprint of LUX gene tagged *A.baumannii*

Trace metals in oily sludge and soil

Trace metals were analysed in oily sludge, which was obtained from experimental site. Trace metals were also analysed in soil, which was collected from different depths of experimental site. Total thirteen metals were analysed in sludge. Out of thirteen metals most of them were within the permissible

limits, except aluminium and iron (Table 2.7) which are non carcinogenic metals.

Similarly out of thirteen metals analysed in soil of experimental site, most of them were within the permissible limit except aluminium and iron (Table 2.7). The test results of trace metals in oily sludge & soil shows that some trace metals such as Si and Ni were absent in sludge but present in soil. Similarly some trace metals such as Zn and Cu were present in sludge but absent in soil of experimental site (Table 2.7) revealed that there is no direct co-relation between trace metals of oily sludge and soil which proves that oily sludge is not the source of trace metals in soil of experimental site. We could not detect trace metals in soil collected at 100 cm depth of experimental plot. Further study is essential in order to monitor trace metals in soil at different depths and in ground water of upstream as well as downstream of treatment site.

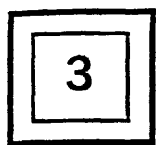
Table 2.7 Trace metals (ppm) in oily sludge and soil of experimental site

Trace metal	Oily sludge	Soil (Zero time)		Soil (After 360 days)	
		25 cm depth	50 cm depth	25 cm depth	50 cm depth
Al	78.6	6.71	53.12	13.30	1.35
Si	---	---	---	---	0.72
Zn	0.60	---	---	---	---
Mn	2.51	0.39	1.35	0.51	0.03
Cu	0.03	---	---	---	---
Co	0.005	0.002	0.006	0.003	0.002
Fe	39.6	10.59	34.87	18.28	1.14
Cd	0.01	0.02	0.02	0.02	0.02
Pb	---	---	---	---	---
Cr	0.04	0.002	0.009	0.004	0.002
Ni	---	---	---	---	0.01
As	0.003	0.001	0.007	0.003	---
Se	---	---	---	---	---

Summary

- Field trial of Oilzapper at Mathura Refinery was initiated in more than 2.5 acre land contaminated with crude oil.
- The study on bioremediation of oily sludge conducted at Mathura Refinery indicates that treatment of oily sludge with Oilzapper (a sludge degrading bacterial system) resulted a reduction of 80% to 96% of oily sludge in contaminated soil.

- Without application of Oilzapper at oily sludge contaminated site resulted only 14% reduction of oily sludge in soil over a period of one year.
- Treatment of oily sludge by Oilzapper is rapid, ecofriendly and cost effective alternative compared to most conventional sludge management practices.
- The cost of per tonne sludge treatment by Oilzapper is only 30-40%, compared to sludge disposal in secured pits.
- Oilzapper also sorbs trace metal and will not allowed to leaching down of trace metals in soil, therefore risks are mitigated.



Field trial of Oilzapper at Barauni refinery

Methods

Selection of site for field trial of Oilzapper at Barauni refinery

Bioremediation of crude oil/oily sludge contaminated soil using Oilzapper has been tested at Barauni refinery. At Barauni Refinery, the site for biodegradation of oily sludge was selected near effluent treatment plant (towards the western side of road No.1) within the boundary wall of plant as shown in Figure 3.1. Initially 4000 m² area was undertaken for biodegradation of oily sludge. The site was marked as sludge treatment site A. Due to encouraging results, additional area was undertaken for sludge biodegradation. The additional area was approximately 2000 m² and marked as sludge treatment site B. At zero time (just before initiation of biodegradation of sludge) soil samples from both treatment sites were collected up to a depth of 30 cm (surface to 30 cm depth). The soil samples were collected by soil sampler. The soil sampler was a 4 cm diameter pipe. The soil of 30 cm horizon (surface to 30 cm depth) was withdrawn by soil sampler and taken in polythene bags and samples were transported to the laboratory at TERI, New Delhi for analysis. The soil samples were analysed for various physical and chemical properties to see the nutrients status of soil. The TPH was also extracted from soil samples collected at zero time to see the hydrocarbon contamination in soil at zero time (before initiation of sludge biodegradation).

Oily sludge loading in treatment site A

Different types of oily sludge such as ETP sludge, crude oil tank bottom sludge and residual sludge (ex-melting pit sludge) were applied in sludge treatment sites at periodical intervals for biodegradation. Heavy dose of Oilzapper and nutrients was applied on oily sludge periodically. The oily sludge and Oilzapper were mixed in soil by tilling of soil with a tractor fitted with cultivator plough. In summer months, water was also sprinkled in treatment sites to maintain the moisture level in soil.

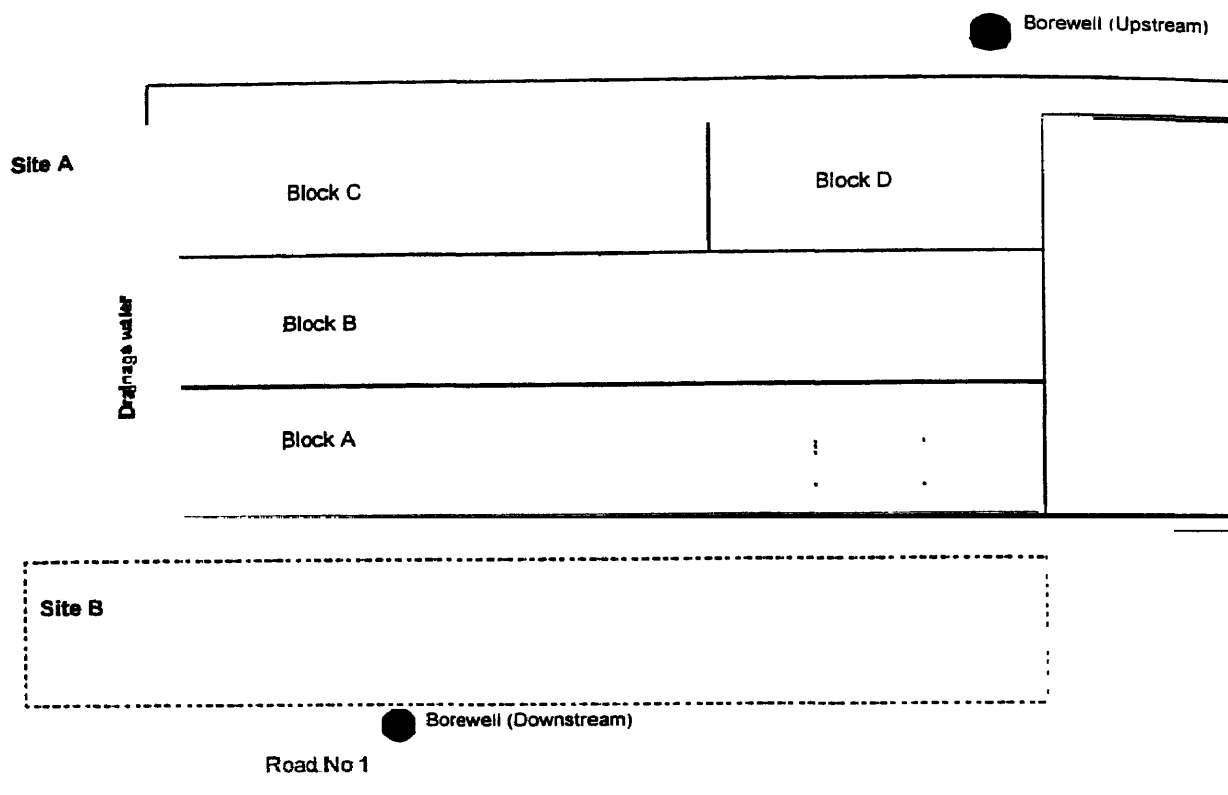


Figure 3.1 Layout of field trial site at Barauni refinery

First sludge loading in treatment site A

Biodegradation of oily sludge with Oilzapper was initiated at Barauni Refinery in the month of May 1998. The sludge loading in treatment site A and B are shown in Table 3.1. Initially in the month of May 1998 approximately 300 tonnes of ETP oily sludge was spread uniformly in 4000 m² area of treatment site A. Oily sludge was mixed in the soil by tilling of soil with a tractor. Based on oily sludge concentration in soil, site A was divided into four blocks. After mixing of sludge in soil, heavy dose of Oilzapper and nutrients was applied over the sludge in treatment site A. An untreated control block (without application of Oilzapper plus nutrients) was also maintained in treatment site A for comparing the biodegradation rates by Oilzapper and indigenous bacterial population present in sludge treatment site A.

After application of Oilzapper and nutrients, soil was again tilled with a tractor for uniform distribution of oily sludge and bacterial cells in soil. Just after mixing of ETP sludge in site A (zero time) soil samples were collected from all the treatment blocks of site A.

Table 3.1 Various types of oily sludge applied in sludge treatment site for biodegradation

Oily sludge applied in treatment site A	Month	Quantity (tonnes)
ETP oily sludge	May 1998	300
Crude oil tank bottom sludge	September 1998	200
Residual oily sludge	December 1998	100
Residual oily sludge	March 1999	300
Oily sludge applied in treatment site B		
Crude tank bottom sludge	December 1998	1500

After mixing of oily sludge and oilzapper plus nutrients in soil, the levels of sludge contamination in soil at zero time (just after sludge loading in treatment site) was estimated. Site A was divided into four blocks and each block was sampled at points of 8 metre distance in straight lines. Each point in straight line was sampled to a depth of 30 cm (surface to 30 cm depth) by soil sampler as described earlier. The soil samples were transported to the laboratory at TERI, New Delhi. Oily sludge was heterogeneously distributed in the soil samples (collected from treatment site A) and therefore, the soil was powdered by soil pulverizer, mixed uniformly by passing through the sieve to uniform distribution of sludge and then TPH from 10 g soil was extracted with hexane, methylene chloride and chloroform consecutively. After extraction all three extracts obtained by hexane, methylene chloride and chloroform were pooled and dried at room temperature by evaporation of solvent under gentle nitrogen stream in fumehood. After solvent evaporation, the amount of TPH recovered from 10 g soil was determined by simple gravimetry.

The next sampling (first observation) in treatment blocks were carried out after 55 days of initial sludge loading in treatment site to see the amount of TPH present in soil after 55 days.

The second observation was taken after 120 days of initial loading of 300 tonnes sludge. The sampling and extraction of TPH from soil samples were identical to that described earlier and the amount of TPH present in treatment site after 120 days was quantified by gravimetry.

Second sludge loading in treatment site A

The second sludge loading in treatment site A was done in the month of September 1998 (Table 3.1). Approximately 200 tonnes of crude tank sludge was spread in 4000 m² area and then oily sludge was mixed in soil by tilling of soil with a tractor. After mixing of tank sludge in soil, heavy dose of oilzapper and nutrients was applied in A, B and C blocks. In control block D, only water

was sprayed. Soil was again tilled for uniform distribution of sludge degrading bacteria. Just After mixing of sludge and sludge degrading bacteria in soil, zero time soil sampling was done as described earlier. Each block was sampled at points of equal distance (8 m distance) in straight line to a depth of 30 cm. The soil from surface to 30 cm depth was withdrawn with a pipe (4 cm diameter) and taken in polythene bags. The soil samples were then transported to the laboratory at TERI, New Delhi. The TPH from 10 g soil was extracted as described earlier and thus the amount of TPH in 10 g soil was estimated just after addition of 200 tonnes sludge in site A. The TPH recovered from 10 g soil was further fractionated into various fractions to quantify the amount of various fraction of sludge in soil.

Treatment site A was sampled after 60 days of addition of 200 tonnes sludge. The sampling and extraction of TPH were identical to that described earlier and thus the amount of sludge recovered from 10 g soil after 60 days of addition of 200 tonnes sludge was estimated. The residual sludge recovered after 60 days was also fractionated and the amount of various fraction of sludge present in soil was quantified by GC. Soil samples collected just after addition of 200 tonnes sludge (zero time) were also analysed for soil bulk density, water holding capacity, organic carbon, nitrogen, available phosphorus potassium, moisture and pH to see the nutrients status of soil.

Third sludge loading in treatment site A

The third sludge loading in treatment site A was done in the month of December 1998. Approximately 100 tonnes residual oily sludge was spread uniformly in site A (4000 m² area). Residual oily sludge was mixed in soil by tilling of soil with a tractor. Heavy dose of oilzapper and nutrients was also applied after mixing of sludge in soil by tilling. The soil was again tilled for uniform distribution of bacterial cells in sludge containing soil. In control blocks, only water was sprayed and tilling was done as carried out in treated blocks. Zero time sampling was done just after mixing of 100 tonnes residual sludge in soil. The sampling was done as described earlier. The TPH was extracted from 10 g soil as described earlier and thus amount of TPH recovered from soil at zero time was quantified. Soil samples collected at zero time were also analysed for soil bulk density, water holding capacity, organic carbon, nitrogen, available phosphorus, potassium, moisture and pH to see the nutrients status of soil.

The soil sampling was also done after 60 days of addition of 100 tonnes residual sludge in treatment site. The soil sampling and extraction of TPH from soil was identical as described earlier. The residual TPH recovered from 10 g

soil was quantified. The residual sludge recovered from soil was further fractionated to quantify the degradation of various fractions of residual sludge.

Fourth sludge loading in treatment site A

The fourth sludge loading in treatment site A was carried out in the month of April 1999. Approximately 300 tonnes residual sludge was spread in 4000 m² area (Table 3.1). The oily sludge was mixed in soil by tilling with a tractor. Heavy dose of oilzapper plus nutrients was applied in treatment blocks. In control block, only water was sprayed. The soil was again tilled with a tractor for uniform mixing of sludge and bacterial cells in soil. The zero time soil sampling was done just after mixing of sludge in soil. The soil sampling and extraction methods were identical as described earlier. At zero time, the amount of TPH recovered from soil of treatment site was quantified. The sampling was also done after 60 days of addition of 300 tonnes sludge and the amount of residual sludge present in soil after 60 days was also quantified. Biodegradation of various fractions of sludge was also quantified in 60 days as described earlier.

Sludge loading in treatment site B

At Barauni Refinery, initially one acre land area (4000 m² area) was undertaken for biodegradation of oily sludge by oilzapper. Due to high biodegradation rate of sludge by oilzapper, additional 2000 m² area (marked sludge treatment site B) situated towards western side of effluent treatment plant (adjacent to road No.1) was undertaken. In the month of December 1998, approximately 1500 tonnes sludge was spread in 2000 m² area (site B) and sludge was mixed in soil by tilling with a tractor. Heavy dose of oilzapper and nutrients was applied just after mixing of sludge in soil. The soil was again tilled for uniform distribution of bacterial cells over the sludge.

Zero time soil sampling (surface 30 cm depth) was done just after sludge loading in treatment site B. The soil sampling and extraction of TPH from collected soil samples were identical as described earlier. To enhance the biodegradation of sludge in site B, the oilzapper and nutrients were also applied at two months interval and soil was also tilled just after application of oilzapper plus nutrients. The sampling from treatment site B was also done at two months intervals. The samples were transported to the laboratory at TERI, New Delhi. The TPH was extracted from samples collected at two months intervals and the amount of residual TPH present in soil of site B was quantified and thus the biodegradation of sludge in site B was estimated.

The residual TPH recovered from soil at different intervals was fractionated into various fractions. The alkane and aromatic fractions were analysed by GLC and thus the amount of various fractions present in residual sludge was quantified and thereby, the biodegradation of various fractions was estimated. Further, to estimate biodegradation of various alkane and aromatic compounds, the alkane fraction was analysed by GC. The aromatic fraction was also analysed by GC and various aromatic compounds were identified.

Survival of the introduced Acinetobacter baumannii strains at field trial site Barauni Refinery.

A.baumannii strains (S19 and S30 constituent of oilzapper) were selected to monitor the survival of the introduced bacterial strains at bioremediation site at Barauni refinery. Both the strains of *A. baumannii* were resistant to different antibiotics and can be easily distinguished from other constituent bacterial strains in the consortium by colony morphology.

Sample Collection from bioremediation site at Barauni Refinery

To monitor the survival of introduced bacterial strains at Barauni refinery soil samples were collected at regular time period from plot A, B, and C. Soil samples were also collected at the end of study from these plots. Similarly soil samples were also collected from plot D at time zero and after completion of study. Soil samples (1 g) from all the four plots were suspended in saline water (0.85% NaCl). The suspensions, after appropriate dilution were plated onto Luria Bertani Agar (LA) containing spectinomycin and ampicilin (50 µg/ml each) in one set of petriplates to estimate the population of *A. baumannii* S19. Similarly the suspension was plated onto LA plates containing 20 µg/ml vancomycin and 50 µg/ml of ampicilin to estimate the population of *A. baumannii* S30. Bacterial colonies (100 colonies) from soil samples of plot A and B were picked randomly from selective LA plates of 10⁵ dilution. Bacterial colonies did not grow from the soil samples of plot D when plated onto selective LA plates (one set of LA plate containing 20 µg/ml vancomycin, 50 µg/ml ampicilin and other set of LA plate containing spectinomycin and ampicilin 50 µg/ml each). These colonies were subjected to Enterobacteriaceae Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) to obtain DNA fingerprints. At time zero the number of colonies picked for ERIC-PCR represented more than 58% of total number of colonies and after one year it represented more than 82% for the total number of colonies. The primers ERIC-1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC-2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3'), were

obtained from Life Technologies, USA. Single isolated colonies were picked at random from the selective LA plates, suspended in 50 µl water and lysed by heating for 10 min at 95 °C. The cell lysate was centrifuged at 12000 revolution per minute (rpm) and 4 °C for 5 minute and 2 µl of the supernatant was used in the reaction mixture. The reaction mixture (15µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.01% gelatin (w/v), 0.2 mM (each) of dNTPs, 1µM each of primers ERIC-1R and ERIC-2 and 0.45 U of Taq polymerase (Life Technologies, USA). The mixture was overlaid with 20 µl mineral oil and amplification was done on DNA engine PTC-200 (MJ Research, USA). The amplification protocol included initial denaturation at 95 °C for 2 min followed by 35 cycles at 92 °C for 30 sec, 50 °C for 1 min.20 sec and 68 °C for 3 min. 20 sec. The final extension was done at 68 °C for 8 min. The reaction was terminated using loading dye (1µl) containing 15% Ficoll, 0.25% bromophenol blue and 0.25% xylene cyanol. Each PCR product was resolved by electrophoresis on a 2% agarose gel. The fingerprint of unknown bacterial isolates thus obtained was compared with the fingerprint of *A. baumannii* strains (S19 and S30) obtained under similar reaction conditions to confirm the survival of the strains at the contaminated site.

Results

In-situ Biodegradation of ETP sludge (First sludge loading) by Oilzapper at Barauni refinery.

At Barauni Refinery, *in situ* biodegradation of sludge with application of oilzapper and nutrients in treatment site A was initiated in the month of May 1998. Initially, approximately 300 tonnes of ETP oily sludge was spread in treatment site A. Based on gradient of TPH concentration in soil, site A was divided into four blocks. Block A, B and C were treated with Oilzapper plus nutrients, while block D was maintained as untreated control block to compare the biodegradation rates of sludge in treated and untreated blocks. Block A treated with oilzapper plus nutrients showed 9.92% contamination of TPH (solvent extractable) in soil just after addition of 300 tonnes ETP sludge and it was reduced to 1.63% and 0.97% after 55 days and 120 days respectively (Figure 3.2). It revealed the biodegradation of 83.5% and 90.2% in 55 days and 120 days respectively (Figure 3.3). In block B at zero time (just after addition of 300 tonnes ETP sludge), 7.61% contamination of TPH (solvent extractable) was recorded. With application of oilzapper and nutrients, the TPH in soil reduced from 7.61% to 1.05% and 0.84% after 55 days and 120 days respectively (Figure 3.2) which accounted for 86.2% and 88.9% biodegradation of sludge in 55 days

and 120 days respectively (Figure 3.3). Block C which was also treated with oilzapper and nutrients showed 6.45%, 3.11% and 0.25% contamination of TPH in soil at zero time (just after addition of 300 tonnes ETP sludge) after 55 days and 120 days respectively (figure 3.2) which accounted for 51.7% and 96% biodegradation of ETP sludge in 55 days and 120 days respectively (Figure 3.3). Block D was maintained as untreated control block showed 3.68% contamination of TPH at zero time (just after loading of 300 tonnes ETP sludge). The contamination of TPH in soil of untreated control block D reduced from 3.68% to 3.26% after 55 days and 3.06% after 120 days (Figure 3.2) which resulted in the biodegradation of sludge by only 11.4% and 16.8% in 55 days and 120 days respectively (Figure 3.3).

In-situ Biodegradation of various fractions of ETP sludge at Barauni Refinery

In blocks treated with oilzapper plus nutrients, alkane and aromatic fractions of oily sludge were biodegraded extensively (Table 3.2). Block A treated with oilzapper plus nutrients showed 83.5% biodegradation of TPH out of which the biodegradation of alkane fraction was accounted for 35%, aromatic fraction was 36% and NSO and asphaltene fraction was 12.5% in 55 days (Table 3.2). In block A, 90.2% TPH was biodegraded in 120 days of which biodegradation of alkane, aromatic and NSO plus asphaltene (combined) fractions were 36%, 39.7% and 14.5% respectively.

Table 3.2 *In situ* biodegradation (%) of various fractions of ETP oily sludge

Blocks	Biodegradation in 55 days				Biodegradation in 120 days			
	TPH	Alkane	Aromatic	NSO + asphaltene	TPH	Alkane	Aromatic	NSO + asphaltene
A	83.5	35.0	36.0	12.5	90.2	36.0	39.7	14.5
B	86.2	36.0	42.0	8.2	88.9	36.0	44.0	8.9
C	51.7	30.9	17.7	3.1	96.0	35.9	44.2	15.9
D	11.4%	10.5	0.5	0.4	16.8	11.8	3.6	1.4

Block B which was treated with oilzapper plus nutrients showed 86.2% biodegradation of TPH in 55 days. The biodegradation of alkane, aromatic and NSO plus asphaltene fractions were 36%, 42% and 8.2% respectively in 55 days (Table 3.3). In the same treatment block, 88.9% of TPH was biodegraded in 120 days of which the biodegradation of alkane, aromatic and NSO plus asphaltene fractions were 36%, 44% and 8.9% respectively (Table 3.2).

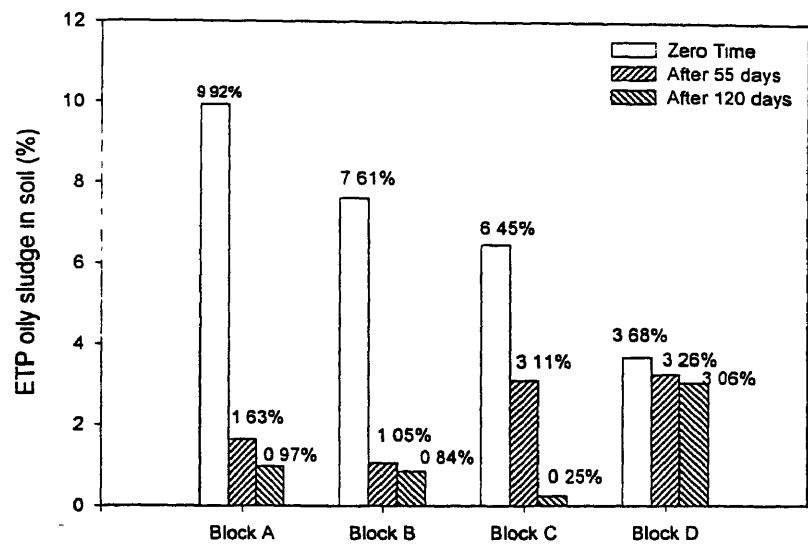


Figure 3.2 ETP oily sludge concentration in soil of field trial Site A

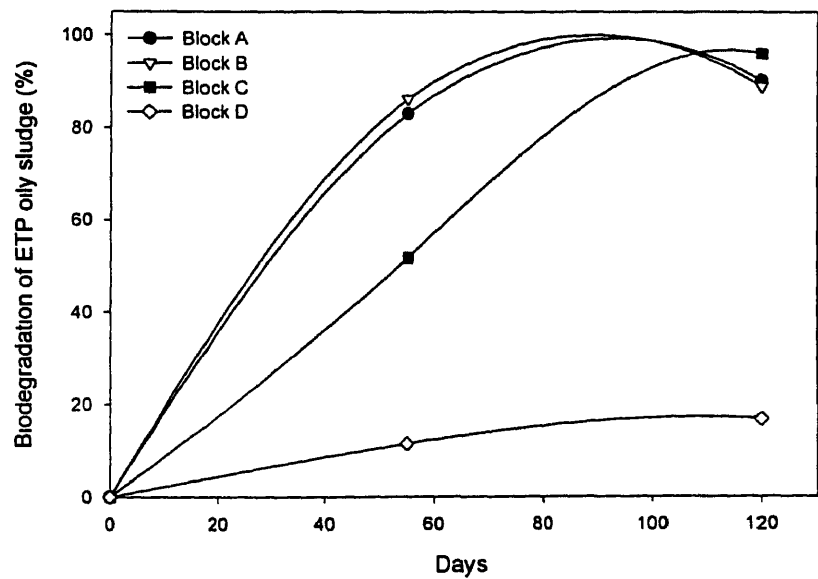


Figure 3.3 Biodegradation of ETP oily sludge (1st sludge loading) in treatment Site A

Note
Blocks A, B and C - Treated with Oilzapper + nutrients, Block D - Uninoculated control

Block C of site A was also treated with oilzapper plus nutrients resulted in 51.7% biodegradation of ETP sludge and 30.9% biodegradation of alkane fraction, 17.7% biodegradation of aromatic fraction and 3.1% biodegradation of NSO and asphaltene (combined) fraction in 55 days. In 120 days, 96% of ETP sludge (solvent extractable TPH) was biodegraded of which the biodegradation of alkane, aromatic, and NSO plus asphaltene fraction were 35.9%, 44.2% and 15.9% respectively (Table 3.2). Block D of site A was maintained as untreated control. In Block D, the biodegradation of ETP and its various fractions were very less as compared to treated blocks (Table 3.2). Among the alkane compounds of ETP sludge, the major biodegradation was of hexadecane, octadecane, eicosane, tetracosane, octacosane and triacotane (Figure 3.4). Biodegradation of aromatic fraction of ETP sludge was also analysed by gas liquid chromatograph. Among the aromatic fractions, polycyclic aromatic hydrocarbon such as acenaphthalene, fluorene, dibenzothiophene, phenanthrene and pyrene (Figure 3.5).

Biodegradation of tank bottom sludge

In treatment site A, approximately 200 tonnes tank bottom sludge was applied for biodegradation. In block A, 1.67% contamination of tank bottom sludge was recorded at zero time (just after additional 200 tonnes tank bottom sludge). With application of oilzapper plus nutrients, the sludge concentration in soil was reduced from 1.67% to 0.56% after 60 days which revealed 66.4% biodegradation of solvent extractable sludge (TPH) in 60 days (Figure 3.6 and 3.7). Similarly, in treatment block B, 0.83% contamination of tank bottom sludge was recorded just after addition of 200 tonnes tank bottom sludge. With application of oilzapper and nutrients, the solvent extractable sludge (TPH) reduced from 0.83% to 0.50% after 60 days which accounted for 37.7% biodegradation of tank sludge (Figure 3.6 and 3.7). However, in block C, the solvent extractable sludge in soil reduced from 0.78% to 0.44% after 60 days which showed 43.5% biodegradation of tank sludge in 60 days (Figure 3.6 and 3.7). In untreated block D, the solvent extractable tank sludge (TPH) contamination in soil reduced from 0.62% to 0.53% after 60 days which resulted in only 14.5% biodegradation of sludge (Figure 3.6 and 3.7). Figure 3.8 shows the treatment site A before and after biodegradation of tank bottom sludge.

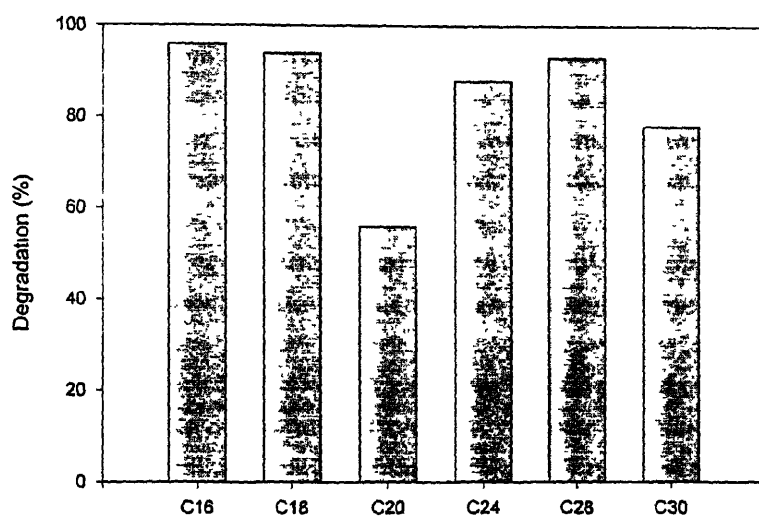


Figure 3.4 Biodegradation of various alkane compounds of oily sludge

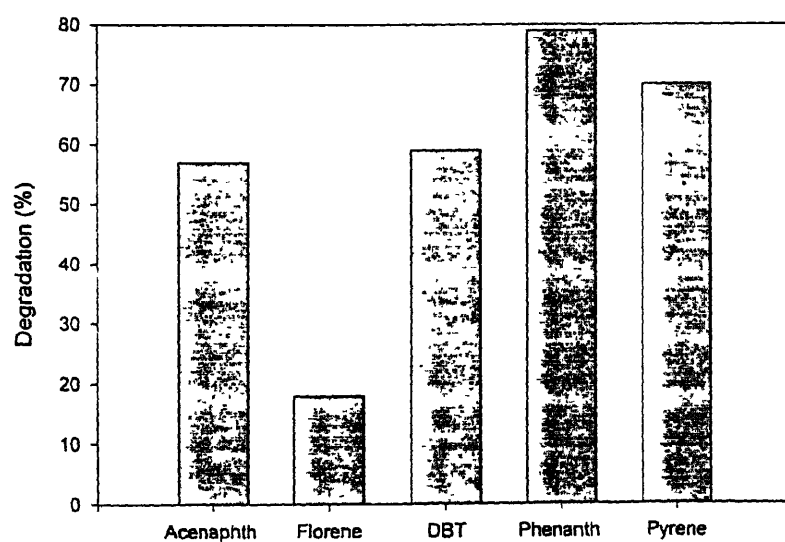


Figure 3.5 Degradation of various PAHs present in oily sludge

Biodegradation of various fractions of tank bottom sludge

In the month of September 1998, approximately 200 tonnes crude oil tank bottom sludge was applied in treatment site A for biodegradation. In block A, 66.4% of solvent extractable tank sludge (TPH) was biodegraded in two months, out of which biodegradation of alkane fraction was accounted for 41%, biodegradation of aromatic fraction of sludge was 18.4% and NSO plus asphaltene fraction was 7% in 60 days (Table 3.3).

In block B, 37.7% biodegradation of tank sludge (solvent extractable) was observed in 60 days of which the biodegradation of alkane, aromatic and NSO plus asphaltene fraction were 25.2%, 9% and 3.5% respectively in 60 days (Table 3.3). Similarly, in block C, 43.5% tank sludge was biodegraded in 60 days.

The biodegradation of alkane fraction of tank sludge was 28%, biodegradation of aromatic fraction of tank sludge was 12% and biodegradation of NSO plus asphaltene fraction was 3.5% in 60 days (Table 3.3). However, in untreated control block D, only 14.5% tank sludge (solvent extractable TPH) was biodegraded in 60 days. In untreated control, the biodegradation of alkane (9%), aromatic (3.6%) and NSO plus asphaltene (1.9%) fractions were very less as compared to treated blocks (Table 3.3).

Table 3.3 *In situ* biodegradation of various fractions of crude oil tank bottom oily sludge by oilzapper in 60 days

Treatment block	Biodegradation %			
	TPH	Alkane	Aromatic	NSO + asphaltene
Block A	66.4	41.0	18.4	7.0
Block C	37.7	25.2	9.0	3.5
Block C	43.5	28.0	12.0	3.5
Block D	14.5	9.0	3.6	1.9

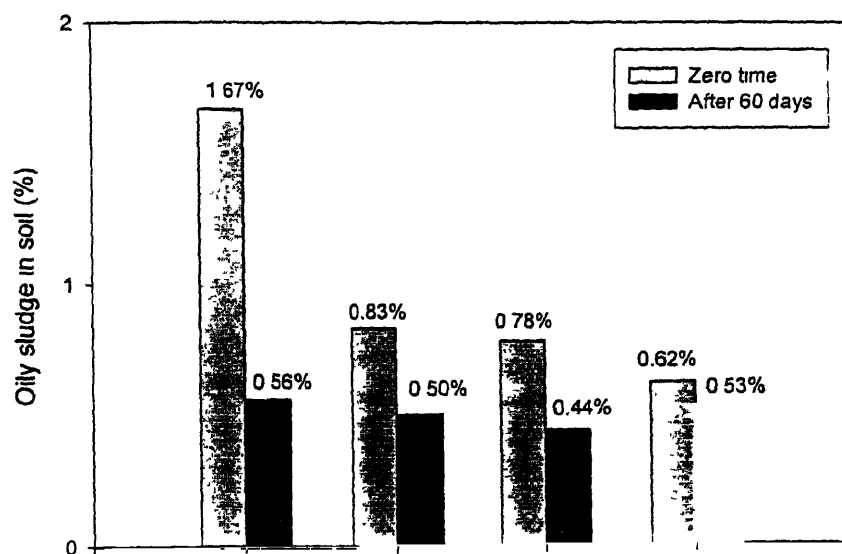


Figure 3.6 Crude tank bottom sludge (TPH) concentration in soil of treatment site A

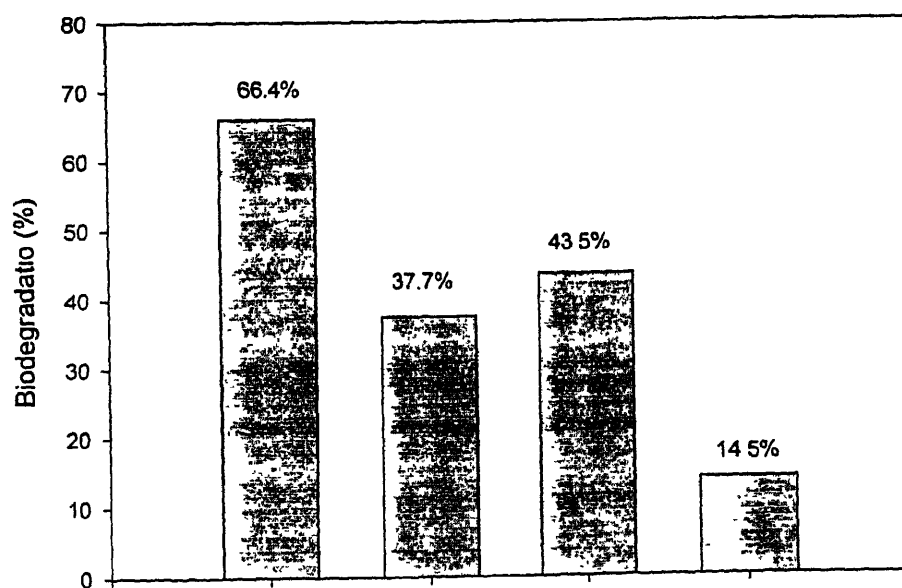
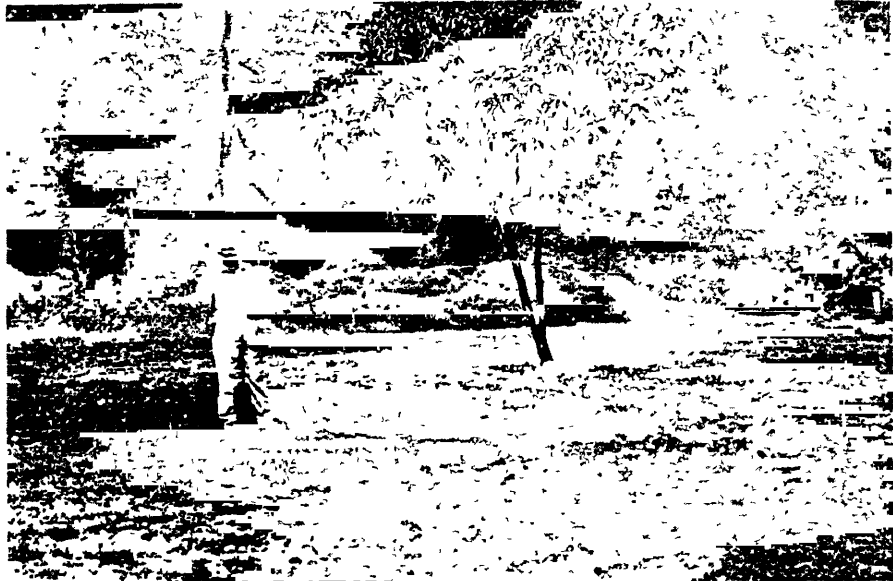


Figure 3.7 In situ biodegradation of crude oil tank bottom sludge (2nd sludge loading) by oilzapper



Before biodegradation of oily sludge



After two months of biodegradation

Figure 3.8 Biodegradation of crude oil tank bottom sludge by oilzapper

Biodegradation of residual sludge

The third sludge loading in treatment site A was carried out in the month of December 1998. Approximately 100 tonnes ex-melting pit sludge (residual sludge after recovery of crude) was applied in treatment site A. Treatment block A of site A was treated with oilzapper plus nutrients showed 4.99% contamination of TPH (solvent extractable residual sludge) in soil at zero time (just after addition of 100 tonnes sludge) and it reduced to 1.51% after 60 days (Figure 3.9) which accounted for 69.7% biodegradation of residual sludge in 60 days (Figure 24). In block B of plot A, 1.86% contamination of TPH (solvent extractable residual sludge) was recorded just after addition of 100 tonnes residual sludge (ex-melting pit). With application of oilzapper and nutrients, the TPH concentration in soil reduced from 1.86% to 0.86% after 60 days (Figure 3.9) which reached 53.7% biodegradation of residual sludge in 60 days (Figure 3.10). Similarly, in block C of site A, 2.69% contamination of residual sludge (TPH) was recorded just after addition of 100 tonnes residual sludge. With application of oilzapper plus nutrients, the residual sludge (TPH) concentration reduced from 2.69 to 0.63 after 60 days (Figure 3.9) which showed 76.5% biodegradation of ex-melting pit sludge in 60 days (Figure 3.10). In untreated control (block D), 2.72% concentration of residual sludge (TPH) was recorded just after addition of 100 tonnes ex-melting pit sludge. In control, the residual sludge concentration in soil reduced from 2.72% to 2.39% after 60 days (Figure 3.9) which revealed only 12.1% biodegradation of sludge in 60 days (Figure 3.10).

Biodegradation of various fractions of residual sludge (sludge after crude recovery) by oilzapper at Barauni refinery

Biodegradation of various fractions of ex-melting pit sludge is shown in Table 3.4. In block A, 69.7% TPH (solvent extractable residual sludge) was biodegraded out of which biodegradation of alkane fraction was accounted for 34.4%, biodegradation of aromatic fraction was 30.5% and NSO plus asphaltene fraction was 4.8% (Table 3.4). Similarly, in block B, which was treated with oilzapper plus nutrients showed 53.7% biodegradation of TPH (solvent extractable residual sludge) in 60 days of which the biodegradation of alkane,

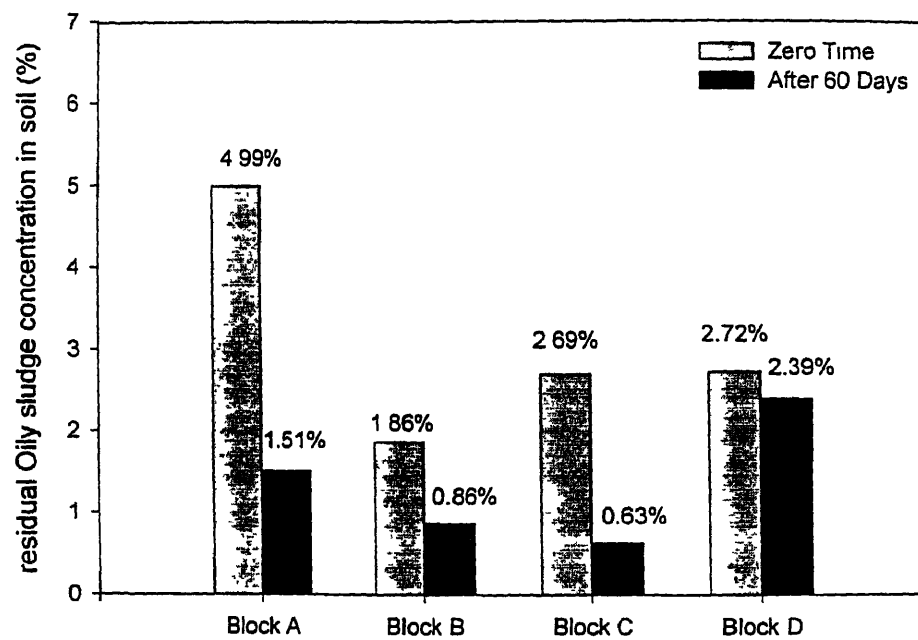
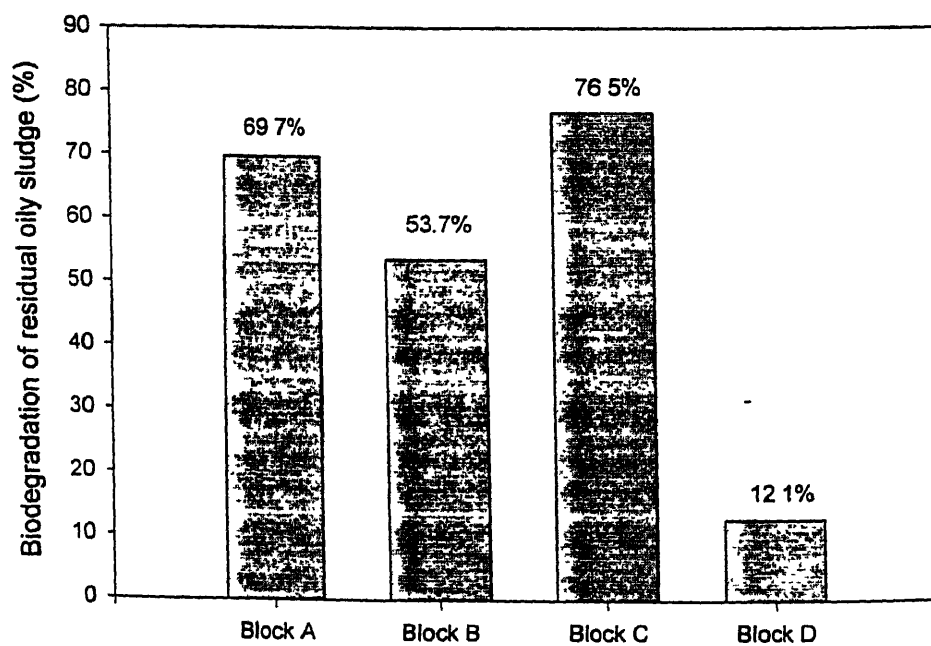


Figure 3.9 Residual oily sludge concentrations in soil of treatment site A

Figure 3.10 Biodegradation of residual oily sludge (3rd sludge loading) in treatment site A

aromatic, and NSO plus asphaltane fractions were 29.8%, 20.2% and 3.7% respectively (Table 3.4). Block C was also treated with oilzapper plus nutrients resulted in 76.5% biodegradation of residual sludge in 60 days. In block C, the biodegradation of alkane fraction was 38.9%, biodegradation of aromatic fraction was 29.1% and biodegradation of NSO plus asphaltane was 8.5% in 60 days (Table 3.4). In control block D, only 12.1% biodegradation of residual sludge was recorded in 60 days of which biodegradation of alkane, aromatic and NSO plus asphaltane fractions were 7.6%, 3.8% and 0.7% respectively in 60 days (Table 3.4).

Table 3.4 *In situ* biodegradation (%) of various fraction of residual oily sludge by oilzapper in 60 days

Treatment blocks	Oily sludge fraction (%)			
	TPH	Alkane	Aromatic	NSO + asphaltane
Block A	69.7	34.4	30.5	4.8
Block B	53.7	29.8	20.2	3.7
Block C	76.5	38.9	29.4	8.5
Block D	12.1	7.6	3.8	0.7

Biodegradation of ex-melting pit sludge at Barauni refinery

The fourth time sludge loading in treatment site A was carried out in March 1999. Approximately 300 tonnes residual sludge was applied in treatment site A. heavy dose of oilzapper plus nutrients was also applied on sludge for biodegradation. In treatment block A, 7.13% concentration of residual sludge (solvent extractable) was recorded in soil at zero time (just after addition of 300 tonnes ex-melting pit sludge). In block A, the residual sludge concentration (TPH) decreased from 7.13% to 1.04% after two months (Figure 3.11) which revealed 85.4% biodegradation of residual sludge in 60 days (Figure 3.12). Block B of site A was also treated with oilzapper plus nutrients showed 6.70% and 2.45% concentration of the residual sludge in soil at zero time (just after addition of 300 tonnes residual sludge) and after 60 days (Figure 3.11) resulted in 63.4% biodegradation of residual sludge in 60 days (Figure 3.12).

In Block C, 7.51% oily sludge concentration was recorded at zero time (just after addition of 300 tonnes residual sludge in treatment site A). With application of oilzapper plus nutrients, the sludge concentration in soil reduced from 7.51% to 2.03% after 60 days (Figure 3.11) resulted in 72.9% biodegradation of residual sludge in 60 days (Figure 3.12). However, in untreated control block D, 8.24% oily sludge concentration in soil was observed

at zero time. After 60 days, the oily sludge concentration in soil reduced from 8.24% to 6.86% (Figure 3.11) indicating only 16.7% biodegradation in 60 days (Figure 3.12).

Biodegradation of various fractions of residual oily sludge (Fourth sludge loading)

In situ biodegradation of various fraction of residual oily sludge (fourth sludge loading in treatment site) in treatment site A is shown in Table 3.5. Treatment block A of site A was treated with oilzapper plus nutrients resulted in 85.4% biodegradation of TPH of fourth sludge loading (300 tonnes residual sludge) in 60 days of which the biodegradation of alkane, aromatic, NSO plus asphaltane fractions was 39.5%, 27.4% and 18.5% respectively in 60 days. In treatment block B, 63.4% oily sludge was biodegraded by oilzapper in 60 days. In same treatment block, the biodegradation of alkane fraction was 34.9%, biodegradation of aromatic fraction was 21.2% and biodegradation of NSO plus asphaltane fraction was 7.3% in 60 days. Similarly, in treatment block C, 72.9% TPH of fourth sludge loading was biodegraded in 60 days of which the biodegradation of alkane fraction was accounted for 37.7%, biodegradation of aromatic fraction was accounted for 24.6% and biodegradation of NSO plus asphaltane fraction was accounted for 10.6% in 60 days. However, in untreated block D, only 16.7% TPH of fourth sludge loading was biodegraded in 60 days. In untreated block D, the biodegradation of alkane (11.8%), aromatic (3.7%), NSO plus asphaltane (1.2%) fractions were also less (Table 3.5).

Table 3.5 *In situ* biodegradation of various fraction of residual (fourth sludge loading) by oilzapper in 60 days

Treatments	Biodegradation (%)			
	TPH	Alkane	Aromatic	NSO + asphaltene
A	85.4	39.5	27.4	18.5
B	63.4	34.9	21.2	7.3
C	72.9	37.7	24.6	10.6
D	16.7	11.8	3.7	1.2

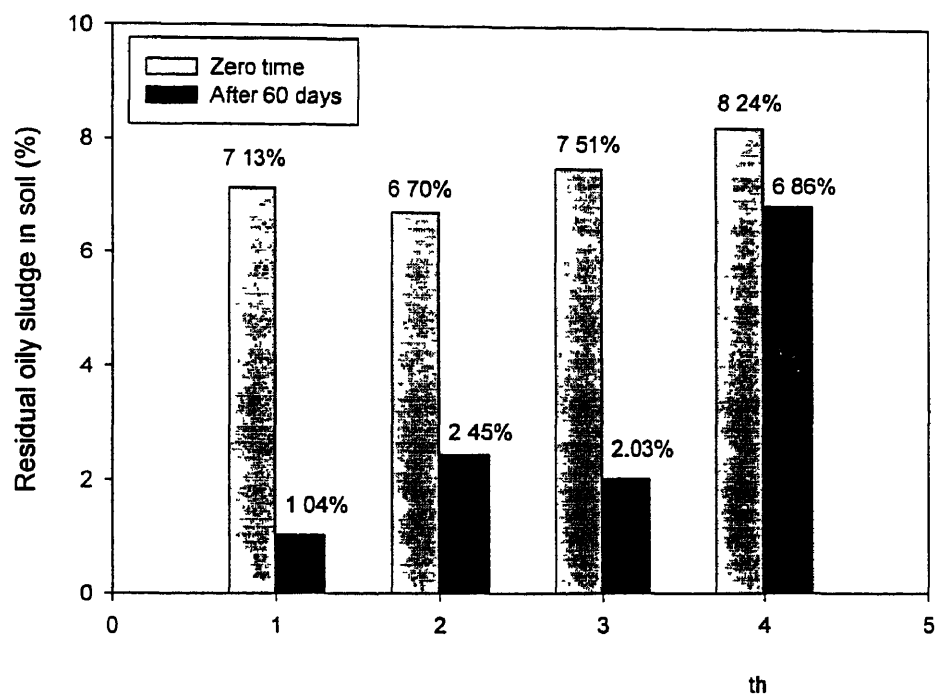


Figure 3.11 Residual oily sludge concentration in treatment site A (4th sludge loading)

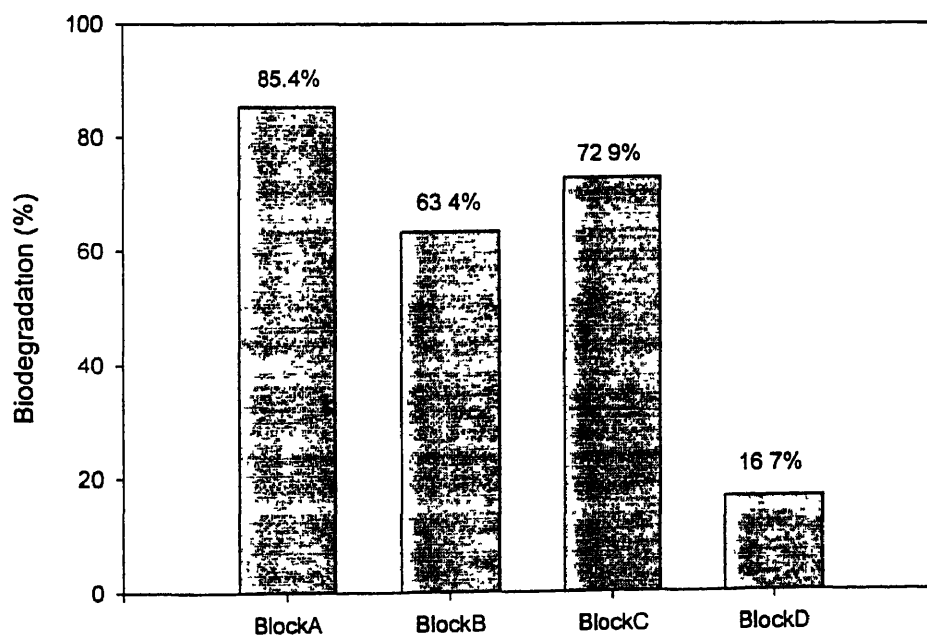


Figure 3.12 Biodegradation of residual oily sludge (4th sludge loading) in treatment site A

Biodegradation of oily sludge at treatment site B

In sludge treatment site B, the biodegradation of oily sludge with application of oilzapper plus nutrients was initiated in the month of December, 1998.

Approximately 1500 tonnes crude oil tank bottom sludge was applied in 2000 m² area of treatment site B. At zero time (just after application of 1500 tonne sludge) 24.9% oily sludge (solvent extractable TPH) concentration was recorded in soil of treatment site B. With application of oilzapper plus nutrients, the oily sludge concentration in soil reduced from 24.9% to 7.7% after 60 days, 4.4% after 120 days and 3.63% after 150 days respectively (Figure 3.13) indicating 69%, 82.3% and 85.4% biodegradation of oily sludge in 60, 120 and 150 days respectively (Figure 3.14). The reclamation of sludge contaminated site after biodegradation of oily sludge are shown in Figure 3.15 and 3.16.

In sludge treatment site B, the alkane and aromatic fractions of oily sludge (TPH) were biodegraded extensively (Table 3.6). With application of oilzapper plus nutrients, 69% of TPH was biodegraded in 60 days of which the biodegradation of alkane, aromatic and NSO plus asphaltene fractions were 39%, 17% and 13% respectively (Table 3.6). With application of oilzapper plus nutrients, 82.3% TPH was biodegraded in 120 days. The biodegradation of alkane fraction was 42%, biodegradation of aromatic fraction was 26.3% and biodegradation of NSO plus asphaltene fraction was 14% in 120 days (Table 3.6). However, in 150 days, 85.4% TPH was biodegraded by oilzapper in sludge treatment site B. The biodegradation of alkane, aromatic and NSO plus asphaltene fractions were 42%, 29.3% and 14.1% respectively in 150 days (Table 3.6).

Table 3.6 In situ biodegradation of various fraction of oily sludge in sludge treatment site B

Oily sludge fraction	Biodegradation (%)		
	60 days	120 days	150 days
TPH	69%	82.3%	85.4%
Alkane fraction	39.0%	42.0%	42.0%
Aromatic fraction	17.0%	26.3%	29.3%
NSO fraction plus Asphaltene	13.0%	14.0%	14.1%

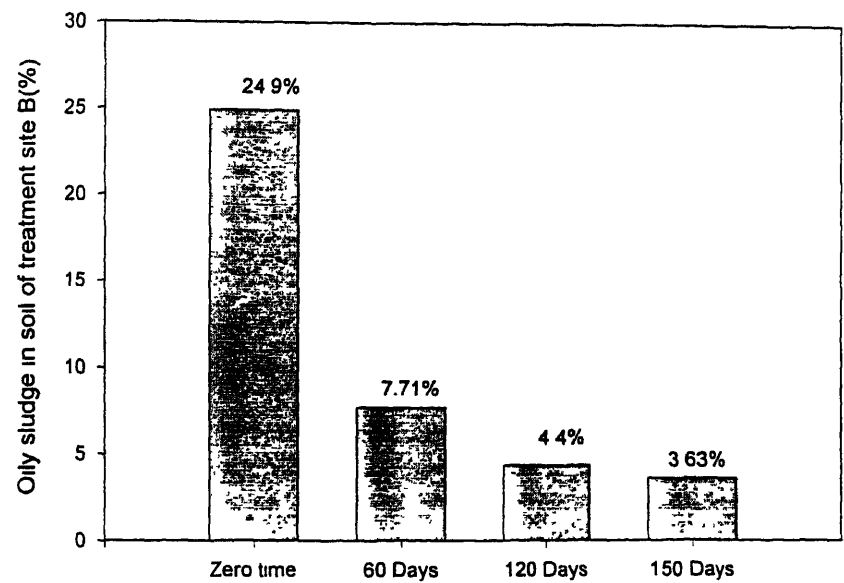


Figure 3.13 Crude tank bottom sludge concentration in treatment site B

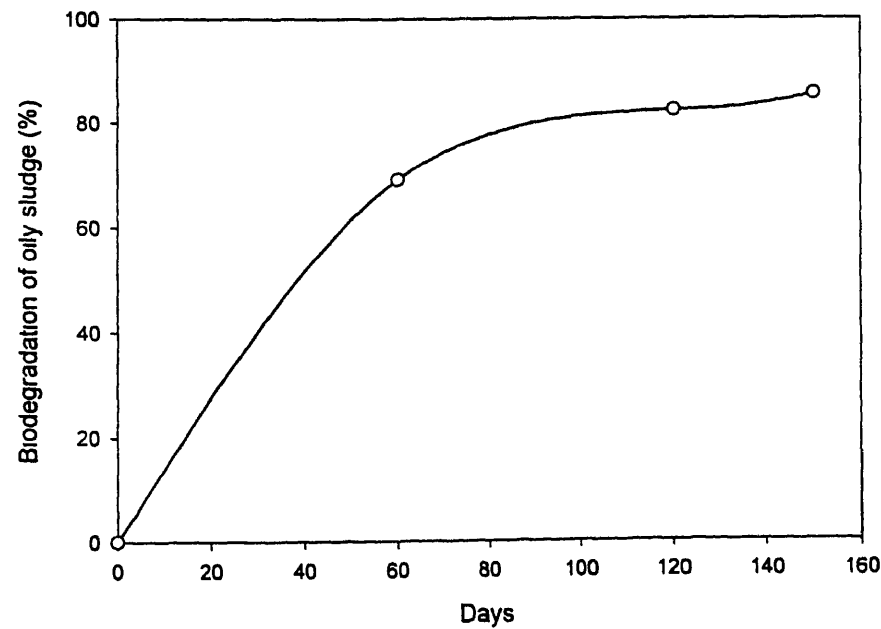


Figure 3.14 Biodegradation of oily sludge in treatment site B



Before biodegradation of oily sludge

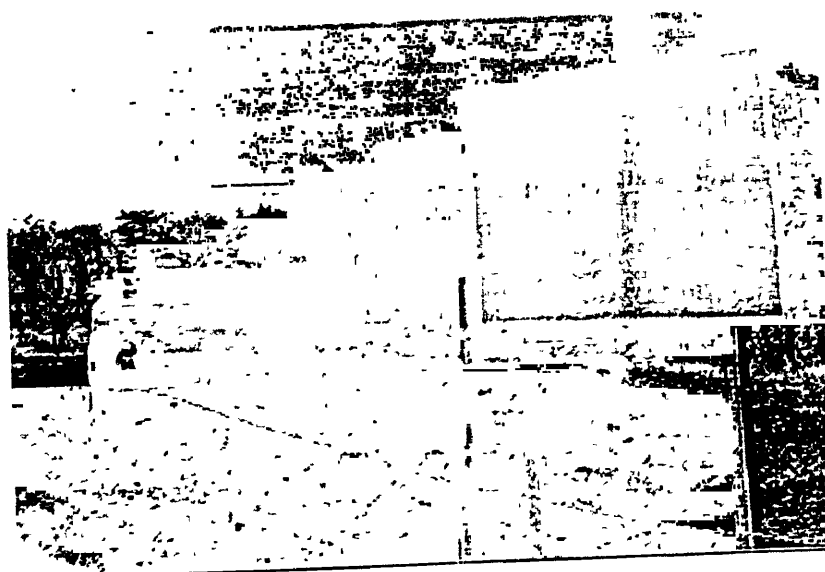


After four months of biodegradation

Figure 3.15 Biodegradation of tank bottom sludge by oilzapper



Before biodegradation



After two months of biodegradation

Figure 3.16 Biodegradation of tank bottom oily sludge by 'Oilzapper'

Survival of Oilzapper at experimental site Barauni Refinery.

A high survivability of *Acinetobacter baumannii* S30 and S19 was found in Barauni refinery. The population of *A. baumannii* in plot A at the start of the study was 1.69×10^7 CFU/g of soil which had reduced to only 1.1×10^7 CFU/g of soil at the end of 120 days. A survivability of 65% was noted. In plot B the population of *A. baumannii* was 2.39×10^7 CFU/g of soil which reduced to 1.32×10^7 CFU/g of soil after 120 days thus depicting a survivability of 55.2%. In plot C the population of *A. baumannii* was 1.85×10^7 CFU/g of soil at zero time and after 120 days the population decreased to 1.27×10^7 CFU/g of soil depicting a survivability of 67.5%. This shows that that reclamation of the contaminated land was possible because of the oily sludge degrading microorganisms present in the selected consortium.

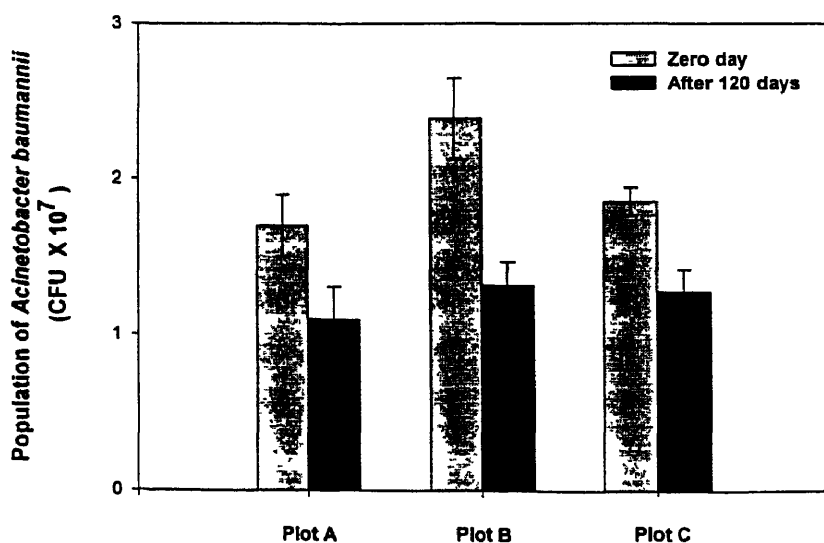


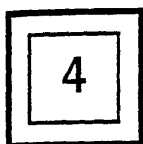
Figure 3.17 Population of introduced *A.baumannii* strains in soil at Barauni refinery

Summary

- The work on field trial of oilzapper at oily sludge contaminated soil was initiated at Barauni Refinery in the month of May 1998. Initially, 4000 m² area (treatment site A) was undertaken for biodegradation of sludge. Approximately 300 tonnes ETP sludge was applied in 4000 m² area (treatment site A). Heavy dose of oilzapper and nutrients were applied in the treatment site for biodegradation. The oily sludge was tilled after application of oilzapper for uniform mixing of sludge and sludge degrading bacterial cells. With application of oilzapper and nutrients, 89% - 96% of oily sludge (solvent extractable TPH) was biodegraded in 120 days. Whereas without application of oilzapper and nutrients, only 17% of oily sludge was biodegraded in 120 days.
- After 4 months of initiation of work, approximately 200 tonnes tank bottom sludge was applied again in same treatment site A (4000 m² area). Treatment with oilzapper and nutrients was continued as explained above. With application of oilzapper plus nutrients, more than 37% to 66% of oily sludge (solvent extractable TPH) was biodegraded in different blocks of treatment site in 60 days. In the absence of oilzapper and nutrients, only 14.1% of oily sludge was biodegraded in 60 days.
- In the month of December 1998 approximately 100 tonnes residual sludge (sludge after recovery of crude) was applied in treatment site A (4000 m² area) for biodegradation. With application of oilzapper plus nutrients more than 53% to 76% of residual sludge was biodegraded in different blocks of treatment site in 60 days. Without application of oilzapper, only 12.1% of oily sludge was biodegraded by indigenous bacteria present at treatment site during the same period.
- In the month of March 1999, approximately 300 tonnes residual sludge was applied in treatment site A. With application of oilzapper plus nutrients, more than 63% to 85% of residual sludge was biodegraded in different treatment blocks in 60 days. However, without application of oilzapper and nutrients, only 16.7% residual sludge was biodegraded by indigenously occurring bacterium (Since in untreated block D, the biodegradation rate was slow, very less quantity of sludge was applied in block D to avoid the accumulation of oily sludge).
- Due to encouraging results, additional area of approximately 2000 m² was undertaken in the month of December 1998 for sludge biodegradation.
- Approximately 1500 tonnes sludge was applied in treatment site B (2000 m² area). Heavy dose of oilzapper and nutrients was applied over the sludge.

With application of oilzapper plus nutrients, more than 85% of residual sludge was biodegraded in treatment site B in 150 days. Thus, in one year, total 2400 tonnes oily sludge was biodegraded by oilzapper in treatment site A and B.

- The TPH and selected toxic metals such as arsenic (As), lead (Pb), chromium (Cr), selenium (Se), cadmium (Cd) and nickle (Ni) could not be detected in soil of experiment site collected from different depths indicating no seepage of TPH and heavy metals in the soil of treatment site.
- Bore wells were installed at upstream and downstream of oily sludge treatment site for underground water pollution monitoring. The TPH could not be detected in upstream and downstream bore well water collected at zero time (just before initiation of experiment), and after one year indicating no seepage of TPH and heavy metals from the sludge treatment site.
- After biodegradation of oily sludge, selected crops plants such as mustard, gram and sunflower were grown in sludge treatment site. The TPH and selected toxic heavy metals could not be detected in tissues of plants grown in sludge treatment site at Barauni Refinery.
- TPH and selected toxic heavy metals could not be detected in the leaves of trees growing in sludge treatment site.



Field trial of Oilzapper at BPCL refinery, Mumbai

Methods

Selection of site for field trial of Oilzapper

Experimental site for *in situ* and on site bioremediation was selected in Tank Dyke of crude tank 115 at BPCL Refinery, Mahul, Mumbai (Figure 4.1). The land in tank dyke was contaminated with tank bottom sludge. Oily sludge in tank dyke was distributed heterogeneously and some spots in tank dyke were saturated with sludge. Oily sludge was mixed in soil of tank dyke by tilling of soil. After mixing of oily sludge in soil, the level of oily sludge contamination in soil at zero time (just before experiment was initiated) was estimated. Initially, the dyke area was divided into four blocks. However, during cleaning of dyke in the month of December, 1999 the sludge from block A was manually removed and dumped into sludge pit. Therefore, the block A was excluded from treatment plot. The blocks B, C and D were sampled at 10 points to a depth of 30 cm before the experiment was initiated and after 60 days of initiation of bioremediation work. Oily sludge was heterogeneously distributed in the soil samples collected from tank dyke, therefore, the soil was powdered by soil pulverizer and mixed uniformly by passing through a sieve to uniformly distribute sludge. Oily sludge from 10 g soil samples collected at zero time (just before initiation of work) was extracted with hexane, methylene chloride and chloroform (100 ml each) consecutively (93-95% extraction efficiency). After extraction all three extracts (obtained by hexane, methylene chloride and chloroform) were pooled and dried at room temperature by evaporation of solvents under gentle nitrogen stream in fume hood. After solvent evaporation, the amount of sludge recovered from 10 g soil was determined by simple gravimetry. The blocks in tank dyke were also sampled at 2 months, 4 months and 6 months intervals. The sampling and extraction of oily sludge from soil samples collected after 2 months, 4 months and 6 months of initiation of work were identical to zero time samples and the amount of total petroleum hydrocarbon (TPH) recovered from soil sample collected after 2 months, 4 months and 6 months were quantified by gravimetry.

Mass scale production of oily sludge degrading bacteria (Oilzapper) and their application at field trial site tank dyke area of tank 115

“Oilzapper” (sludge degrading bacterial system) was grown in a fermenter on minimal salt medium. After 16 hours of growth cultures were harvested and mixed with a carrier material. The carrier based Oilzapper (containing 10^{10} cells/g carrier material) was filled in sterilized poly bags. Poly bags containing Oilzapper were sealed and kept in dark at 25°C for 7 days for curing. Cured culture packets were transported to BPCL Refinery, Mumbai and applied in experimental sludge treatment site.

Oily sludge was distributed heterogeneously in experimental site (tank dyke area of tank 115), therefore, the site was divided into three blocks. “Oilzapper” and nutrients were applied in experimental blocks B and D. In block C, only nutrients were applied. Soil in block B, C and D were tilled for uniform distribution of bacterial cells.

Soil samples from bioremediation site collected at zero time were analyzed for soil bulk density, water holding capacity, organic carbon, nitrogen, available phosphorus, potassium, moisture and pH to see the nutrient status of soil. The soil samples of treatment site would also be analysed after completion of bioremediation work to see the improvement in soil conditions.

Analysis of oily sludge biodegraded at BPCL refinery

Oily sludge that was lying in tank dyke of tank 115 was analysed before initiation of work. The total petroleum hydrocarbon (TPH) was extracted from oily sludge using hexane, methylene chloride and chloroform. After evaporation of solvents the amount of TPH recovered from 10g oily sludge was quantified. The TPH that was extracted from oily sludge was further fractionated into alkane, aromatic, asphaltene and NSO fractions (Nitrogen, Oxygen, and sulphur containing compound) with a silica gel column. The ash content of oily sludge was estimated after extraction of TPH from oily sludge. A known amount of oily sludge was taken in crucible and heated at 600°C for five hours. After cooling the amount of ash recovered from 10g sludge was quantified by gravimetry.

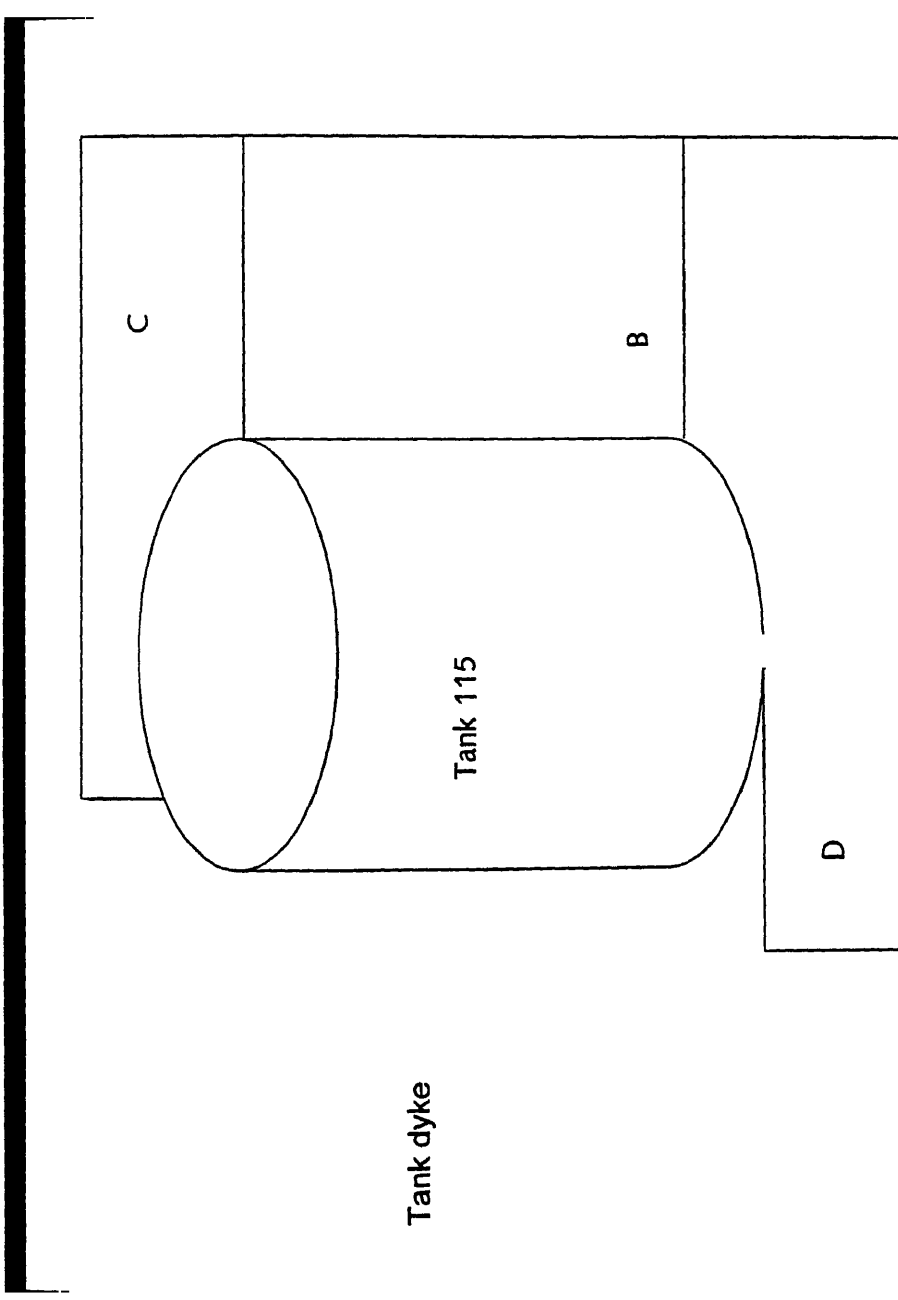


Figure 4.1. Layout of bioremediation site at tank farm area of tank 115

Composition of TPH recovered from oily sludge

The TPH recovered from oily sludge at zero time and after 2, 4 and 6 months were fractionated into alkane, aromatic asphaltene and NSO (nitrogen, oxygen, and sulphur containing compound) with a silica-gel column. The TPH was first dissolved in n-pentane and separated into soluble and insoluble fractions (asphaltene). The soluble fraction was loaded on silica-gel column and eluted with different solvents. The alkane fraction was eluted with hexane, followed by the aromatic fraction that was eluted with benzene. The NSO fraction was eluted with chloroform and methanol. Alkane and aromatic fractions were concentrated by evaporation of solvents and then 0.2 µl of each was analyzed by gas chromatography (GC Hewlett Packard, 5890 Series II) to identify all the compounds present in the alkane and aromatic fractions. In the alkane fraction, various alkane compounds were identified by matching the retention time with authentic standards. Simultaneously, the identification of each alkane compound was also confirmed by spiking the alkane fraction. A known amount of each standard alkane compound was added to the alkane fraction and injected into GC (co-injection) and the peak area of each alkane compound increased as a result. Similarly, the polycyclic aromatic hydrocarbon compounds were identified by matching the retention time with an authentic standard and confirmed by spiking the sample with authentic standards.

Results

Analysis of soil collected from field trial

Before initiation of bioremediation work at BPCL Refinery, Mumbai, the soil of 30 cm horizon (surface to 30 cm depth) was collected from the tank dyke of tank 115 using soil sampler. Zero time soil analysis of experimental site showed 4.8% organic carbon, 62 ppm available phosphorus and 175 ppm potassium, 0.08% total nitrogen, 52% water holding capacity, pH of 7.77 and soil bulk density of 0.99g/cm³ with black as the soil texture (Table 4.1).

After completion of bioremediation of oily sludge in tank dyke of tank 115, the soil characteristics at bioremediation site has improved. At zero time, the organic carbon in soil of bioremediation site was high (4.8%) due to oily sludge contamination in soil. After bioremediation of oily sludge, the organic carbon in soil reduced from 4.8% to 2.6%. The total nitrogen in soil at bioremediation site was increased from 0.08% to 0.09% (Table 1). At zero time, the water holding capacity and bulk density of soil at bioremediation site was less as compared to normal agricultural land due to oily sludge contamination in soil. After

bioremediation of oily sludge, the water holding capacity and soil bulk density has improved to normal agricultural land (Table 4.1).

Table 4.1 Physical and Chemical properties of soil of experimental site

Tests	Results	
	Zero time	After six months
Texture	Black	Brown
Bulk density	0.99 gm/cm ³	1.2 gm/cm ³
Water holding capacity	52%	65%
pH	7.77	7.65
Organic carbon	4.8%	2.6%
Total nitrogen	0.08%	0.09%
Potassium	175 ppm	165 ppm
Available phosphorus	62 ppm	72 ppm

Composition of oily sludge biodegraded at BPCL refinery

The composition of oily sludge which was lying in tank dyke of crude tank 115 was analysed. Oily sludge on extraction shows solvent extractable TPH 78%, sediments 9% and 13% water content (Table 4.2). The extractable TPH obtained from oily sludge was further fractionated into various fractions showed 67% alkanes, 20% aromatics, 11% asphaltene and 2% NSO (nitrogen, oxygen, sulphur containing compounds) as shown in Table 4.3.

Table 4.2 Characterization of oily sludge of crude tank 115

Composition of oily sludge	Results
Total petroleum hydrocarbon (Solvent extractable)	78%
Sediments/Ash	9%
Water	13%

Table 4.3 The composition of total petroleum hydrocarbon extracted from oily sludge of crude tank No.115

Fractions	Percent (%)
Alkane (Saturated fraction)	67%
Aromatic	20%
Asphaltene	11%
NSO	2%

In-situ bioremediation of oily sludge at BPCL refinery

Oily sludge contamination levels in soil of treatment blocks of B, C and D are shown in Figure 4.2. Block C and D were treated with Oilzapper plus nutrient, while Block C was maintained as control which was treated with nutrient alone.

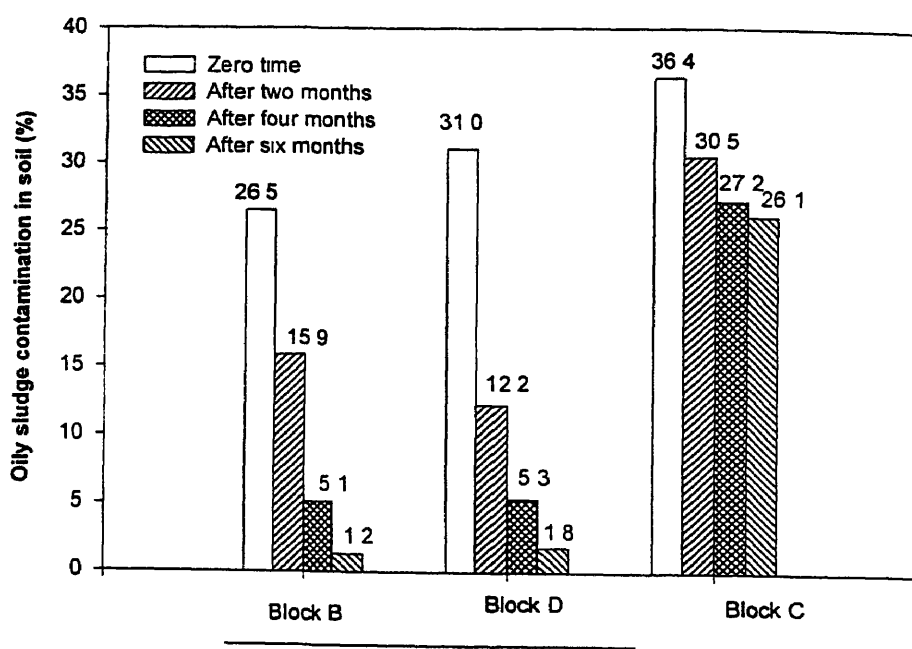
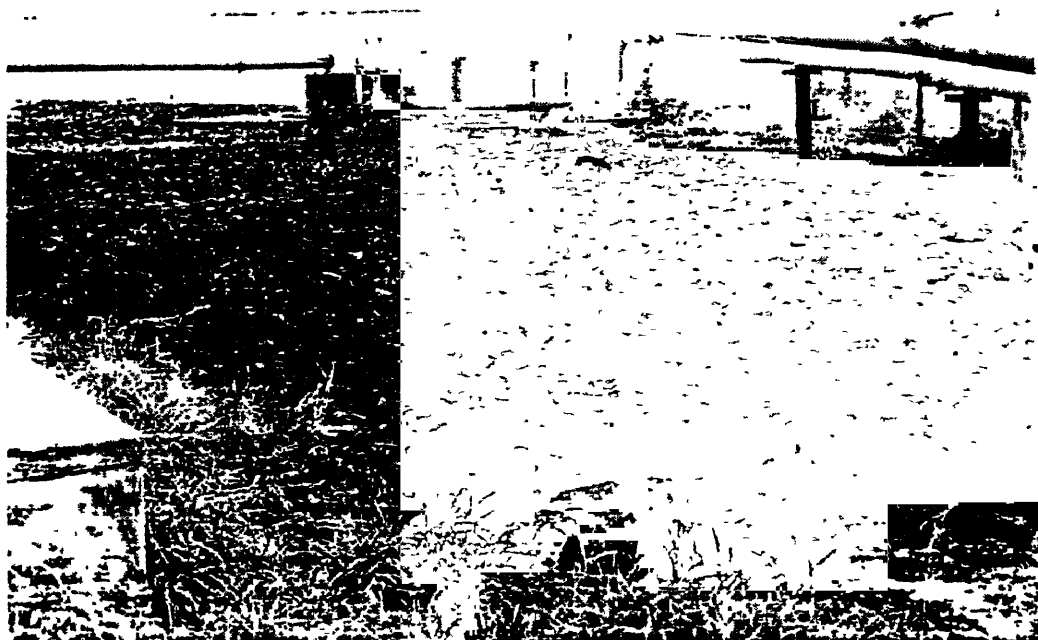


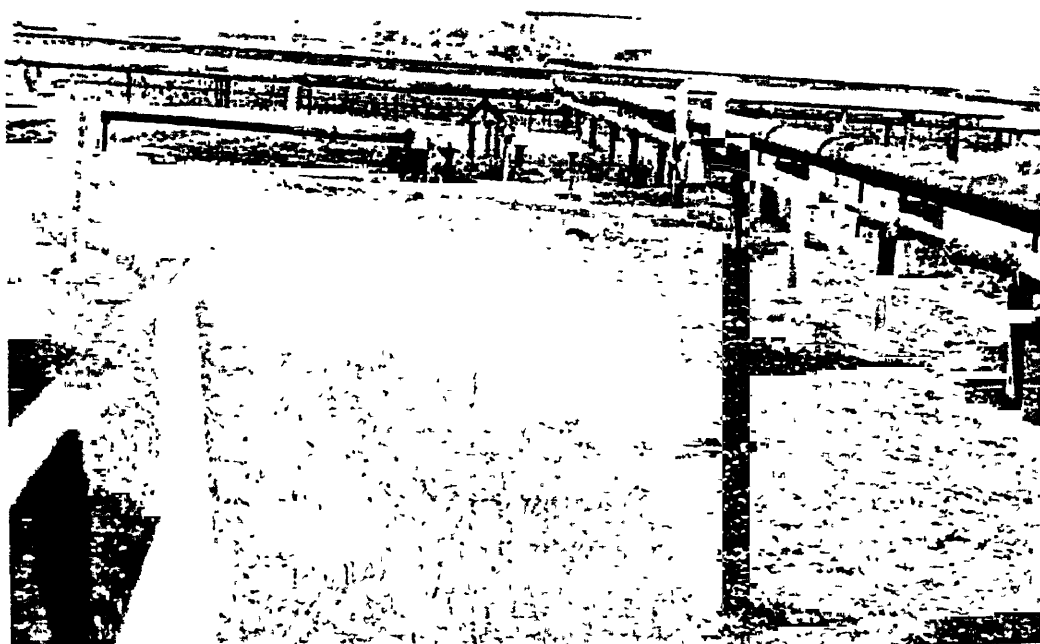
Figure 4.2 Biodegradation of oily sludge by oilzapper at BPCL refinery, Mumbai

At zero time (just before initiation of work), the oily sludge (TPH) contamination in block B was 26.5%. With application of Oilzapper plus nutrient, the oily sludge (TPH) contamination reduced from 26.5% to 1.2% in six months which revealed 95.4% biodegradation of oily sludge in six months. With application of Oilzapper and nutrients in block B, the oily sludge (TPH) contamination in soil of block B after two and four months was 15.9% and 5.1% respectively showing biodegradation of 40% and 80.7% respectively (Figure 4.2). Similarly, in block D, the oily sludge concentration recorded was 31.0% at zero time. With application of Oilzapper and nutrient, the sludge concentration reduced from 31.0% to 1.8% which accounted for 94.1% biodegradation of sludge in six months. The oily sludge contamination (solvent extractable TPH) in soil of block D at bioremediation site after two months and four months of

initiation of bioremediation was 12.2% and 5.3% revealing biodegradation of 60.6% and 82.9% respectively (Figure 4.2). However, block C was maintained as control (treated only with nutrients) showed the oily sludge contamination reduced from 36.4% to 26.1% in six months which accounted for only 28.2% biodegradation of sludge in control block. The sludge contamination in soil of control block C (treated with nutrients alone) after two and four months was 30.5% and 27.2% showing a loss of 16.2% and 25.2% respectively which may be due to indigenous micro-organisms present at bioremediation site (Figure 4.2). After bioremediation of oily sludge the soil has become normal. The sludge toxicity has also reduced after biodegradation of oily sludge (Figure 4.5).



Before bioremediation of oily sludge



After two months of bioremediation of oily sludge

Figure 4.3 Oily sludge bioremediation site in tank farm area of tank 115

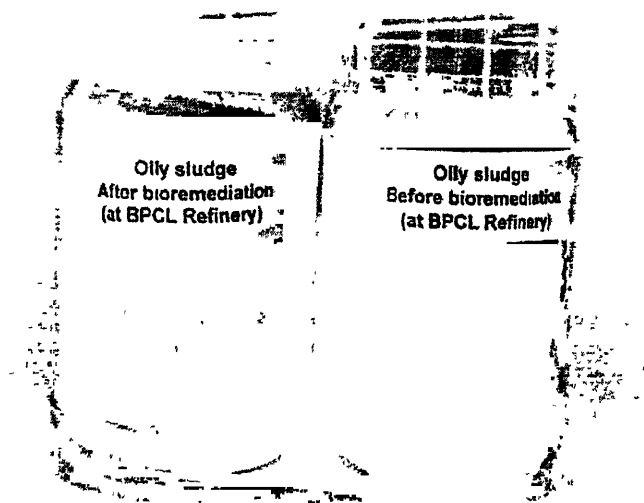
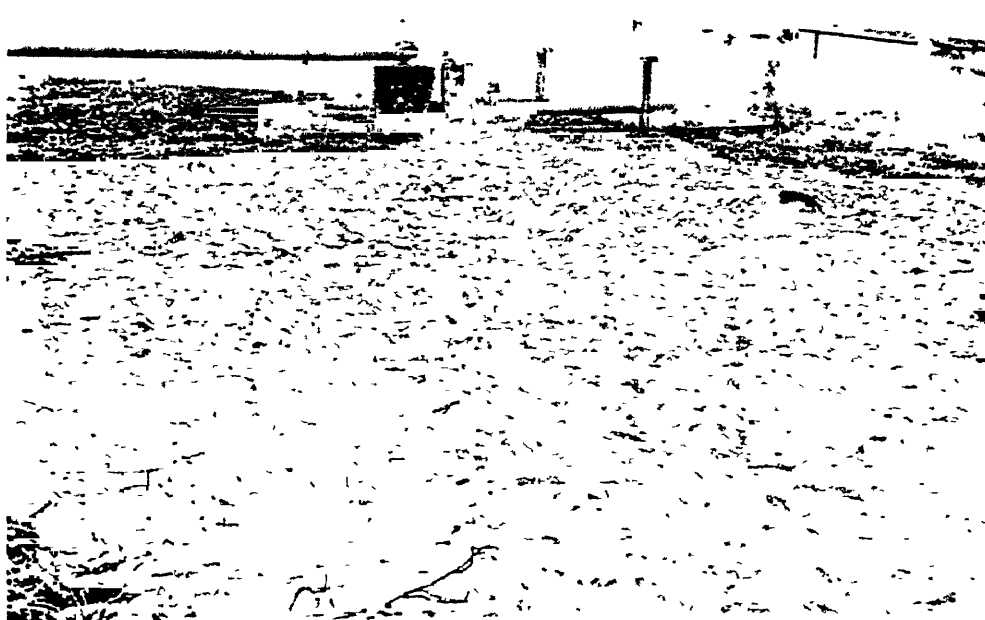


Figure 4.4 Bioremediation of tank bottom sludge by Oilzapper - an oily sludge degrading bacterial system

Bioremediation of various fractions of oily sludge

Bioremediation of various fractions of oily sludge at bioremediation site was estimated. Block B in tank dyke of tank 115 was treated with Oilzapper (oily sludge degrading bacteria) plus nutrients showed 95.4% biodegradation of oily sludge in six months of which alkane fraction was accounted for 66.1%, aromatic fraction 19.4% and NSO plus asphaltene fraction 9.9% (Table 4.4). Similarly, biodegradation of various fractions of oily sludge in two months and in four months is also shown in Table 4. GC fingerprinting of biodegradation of alkane and aromatic fraction in block B is shown in Figure 7 and 8 respectively. Block D was also treated with Oilzapper plus nutrients revealed 94.1% biodegradation of oily sludge (TPH) in six months. Out of 94.1% biodegradation of oily sludge, the biodegradation of alkane, aromatic and NSO plus asphaltene fraction was accounted for 66.4%, 19.2% and 8.5% respectively (Table 4.5).



Before bioremediation of oily sludge



After bioremediation of site

Toxicity of sludge has reduced and grasses sprouted in treated site)

Figure 4.5 Bioremediation of oily sludge and reduction of sludge toxicity

Table 4.4 Biodegradation of various fractions of oily sludge in block B at bioremediation site in tank dyke of tank 115

Oily sludge fraction	Bioremediation (%)		
	In two months	In four months	In six months
Total petroleum hydrocarbon	40.0	80.7	95.4
Alkane fraction	26.5	56.3	66.1
Aromatic fraction	11.6	19.1	19.4
NSO + Asphaltene fraction	1.9	5.3	9.9

Table 4.5 Biodegradation of various fraction of oily sludge in block D at bioremediation site in tank dyke of tank 115

Oily sludge fraction	Bioremediation (%)		
	In two months	In four months	In six months
Total petroleum hydrocarbon	60.6	82.9	94.1
Alkane fraction	44.8	58.7	66.4
Aromatic fraction	12.5	15.6	19.2
NSO + Asphaltene fraction	3.3	8.6	8.5

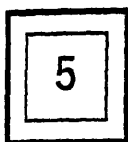
Block C was treated only with nutrients alone exhibited only 28.2% biodegradation of oily sludge in six months. In block C, biodegradation of alkane, aromatic, NSO plus asphaltene fraction in 2, 4 and 6 months were also less (Table 4.6) as compared to biodegradation of these fractions in block B and D which were treated with Oilzapper plus nutrients.

Table 4.6 Biodegradation of various fractions of oily sludge in block C at bioremediation site in tank dyke of tank 115

Oily sludge fraction	Bioremediation (%)		
	In two months	In four months	In six months
Total petroleum hydrocarbon	16.2	25.3	28.2
Alkane fraction	8.3	16.8	18.4
Aromatic fraction	6.9	7.5	8.1
NSO + Asphaltene fraction	1.0	1.0	1.7

Summary

- The field trial of oily sludge by oily sludge degrading bacterial system Oilzapper was initiated in the month of October 1999 in the tank dyke of crude tank 115 at BPCL refinery.
- Bioremediation site (in tank farm area) was divided into block B, C and D. Blocks B and D were treated with Oilzapper plus nutrient, while block C was treated only with nutrient.
- Block B was treated with Oilzapper plus nutrients showed that oily sludge in soil of tank farm area reduced from 26.5% to 1.2% in six months which revealed 95.4% biodegradation of oily sludge in six months.
- Block D was also treated with Oilzapper plus nutrients exhibited that the oily sludge concentration reduced from 31.0% to 1.8% in six months which indicated 94.1% biodegradation of sludge in six months.
- In control block C, oily sludge concentration reduced from 36.4% to 26.1% in six months which resulted in only 28.2% biodegradation of sludge in six months.
- In block B, more than 98% alkane fraction and aromatic fraction of oily sludge have been biodegraded by Oilzapper in six months. Similarly, in block D, more than 96% alkane fraction and aromatic fractions of oily sludge have been biodegraded in six months. However, in untreated control block, only 29% of alkane fraction and only 40% of aromatic fraction of oily sludge have been biodegraded in six months.



Field trial of oilzapper at Digboi refinery

Methods

Selection of site for field trial of oilzapper at Digboi refinery

Experimental site for *in situ* and on site bioremediation was selected in Tank Dyke of crude tank A305 at Digboi Refinery. The land in tank dyke was contaminated with tank bottom sludge. Oily sludge in tank dyke was distributed heterogeneously and some spots in tank dyke were saturated with sludge. Oily sludge was mixed in soil of tank dyke by tilling of soil. After mixing of oily sludge in soil, the level of oily sludge contamination in soil at zero time (just before experiment was initiated) was estimated. The dyke area was divided into four blocks. The blocks A, B, C were treated with Oilzapper plus nutrients while block D was treated only with nutrients. Each block was sampled at 10 points to a depth of 30 cm before the experiment was initiated and after 60 days of initiation of bioremediation work. Oily sludge was heterogeneously distributed in the soil samples collected from tank dyke, therefore, the soil was powdered by soil pulverizer and mixed uniformly by passing through a sieve to uniformly distribute sludge. Oily sludge (total petroleum hydrocarbon – TPH) from 10 g soil samples collected at zero time (just before initiation of work) was extracted with hexane, methyle chloride and chloroform (100 ml each) consecutively (93-95% extraction efficiency). After extraction all three extracts (obtained by hexane, methylene chloride and chloroform) were pooled and dried at room temperature by evaporation of solvents under gentle nitrogen stream in fume hood. After solvent evaporation, the amount of sludge (TPH) recovered from 10 g soil was determined by simple gravimetry. The blocks in tank dyke were also sampled at 60 days intervals of initiation of experiment. The sampling and extraction of oily sludge (TPH) from soil samples collected after 60 days of initiation of work were identical to zero time samples and the amount of sludge recovered from soil sample collected after 60 days was quantified by gravimetry. Thus, the amount of sludge (TPH) biodegraded by Oilzapper in two months was estimated by difference of sludge present in treatment blocks at zero time and after two months.

Mass scale production of oilzapper and its application at field trial site tank dyke area of tank A305 and ETP area

“Oilzapper” (a sludge degrading bacterial system) was grown in a fermenter on minimal salt medium. After 16 hours of growth cultures were harvested and mixed with a carrier material. The carrier based Oilzapper (containing 10^{10} cells/g carrier material) was filled in sterilized polythene bags and sealed. Sealed culture packets were kept in dark at 25°C for 7 days for curing. Cured culture packets were transported to Digboi Refinery and applied in experimental sludge treatment site.

Analysis of Oily sludge

Oily sludge that was lying in sludge drying pit was analysed before initiation of work. The total petroleum hydrocarbon (TPH) was extracted from oily sludge using hexane, methyle chloride, and chloroform. After evaporation of solvents the amount of TPH recovered from 10g sludge was quantified. The TPH that was extracted from oily sludge was further fractionated into alkane, aromatic, asphaltene and NSO fractions (Nitrogen, Sulphur, and Oxygen containing compound) with a silica gel column. The ash content of oily sludge was estimated after extraction of TPH from oily sludge. A known amount of oily sludge was taken in crucible and heated at 600°C for five hours. After cooling the amount of ash recovered from 10g sludge was quantified by gravimetry.

Biodegradation of various fractions of oily sludge

The TPH recovered from oily sludge at zero time and after two months were fractionated into alkane, aromatic asphaltene and NSO (nitrogen, sulphur, and oxygen containing compound) with a silica-gel column. The TPH was first dissolved in n-pentane and separated into soluble and insoluble fractions (asphaltene). The soluble fraction was loaded on silica-gel column and eluted with different solvents. The alkane fraction was eluted with hexane, followed by the aromatic fraction that was eluted with benzene. The NSO fraction was eluted with chloroform and methanol. Alkane and aromatic fractions were concentrated by evaporation of solvents and then 0.2 µl of each was analyzed by gas chromatography (GC Hewlett Packard, 5890 Series II) to identify all the compounds present in the alkane and aromatic fractions. In the alkane fraction, various alkane compounds were identified by matching the retention time with authentic standards. Simultaneously, the identification of each alkane compound was also confirmed by spiking the alkane fraction. A known amount of each standard alkane compound was added to the alkane fraction and

injected into GC (co-injection) and the peak area of each alkane compound increased as a result. Similarly, the polycyclic aromatic hydrocarbon compounds were identified by matching the retention time with an authentic standard and confirmed by spiking the sample with authentic standards.

In-situ Bioremediation of oily sludge in ETP area at Digboi Refinery

In ETP area, three sites were selected for bioremediation of oily sludge. Site A, B and C were situated towards upper side of sludge drying bed in ETP area. In site A, approximately 100 kg sludge was loaded for bioremediation while site B and C were containing sludge (sludge dumping sites). All three sites were treated with Oilzapper plus nutrients.

Oily sludge in all the three sites were mixed uniformly by tilling of sites. After mixing of sludge by tilling, the sites A, B and C were sampled at 10 points. The samples were collected in polythene bags and were transported to the laboratory at TERI, New Delhi for analysis. The oily sludge (TPH) from 10 g samples were extracted with hexane, methylene chloride and chloroform (100 ml each) consecutively as described earlier. The TPH recovered from 10 g samples were fractionated into various fractions by silica gel column as described earlier and each fraction was analysed by GC. The sampling and extraction of sludge (TPH) from samples collected from site A, B and C after 60 days of initiation of work were identical to zero time samples and thus amount of sludge recovered from samples collected after 60 days was quantified by gravimetry.

In-situ Bioremediation of oily sludge in sludge drying pit

Sludge drying pit 6 was undertaken for bioremediation of oily sludge.

Approximately 100 kg Oilzapper was applied in sludge pit and more than 100 litre nutrients mixture was also sprayed on oily sludge. Compressed air was also blown in sludge drying pit 6 during night time to provide air to the bacterial cells. At zero time, sludge was collected from six spots in sludge pit. The solvent extractable total petroleum hydrocarbon (TPH) from sludge samples collected at zero time was estimated. Sludge samples was also collected after two months of initiation of bioremediation work and solvent extractable TPH was estimated. The difference in TPH content of sludge samples of zero time and after two months was estimated and thus the biodegradation of TPH in sludge drying pit was estimated. The TPH recovered from sludge of sludge drying pit 6 at zero time and after two months was fractionated into various fractions and their

concentration was estimated. The alkane and aromatic fractions were analysed by GC.

Results

Composition of oily sludge of sludge biodegraded in drying pit

The composition of sludge of sludge drying pit No.6 was analysed. The solvent extractable total petroleum hydrocarbon (TPH) was 51.14%, water 22.6%, and organic matter 18% and sediments 10.54% (Table 5.1).

Table 5.1 Composition of oily sludge of sludge drying pit No.6

Composition of oily sludge	Results
Total petroleum hydrocarbon (solvent extractable)	51.14%
Water in sludge	22.60%
Organic matter	18%
Sediments	10.54%

Values are mean of six replicates

The TPH recovered from oily sludge of sludge drying pit 6 was further fractionated into various fractions which revealed 54% alkane fraction, 38% aromatic fraction, 2% NSO fraction and 6% asphaltene fraction (Table 5.2).

Table 5.2 Composition of total petroleum hydrocarbon from oily sludge of sludge drying pit No.6

Fractions	Percent (%)
Alkane (saturated fraction)	54%
Aromatic fraction	38%
NSO fraction	2%
Asphaltene fraction	6%

In-situ Biodegradation of oily sludge in tank dyke of tank A 305

Oily sludge contamination levels in treatment blocks of A, B, C and D are shown in Figure 5.1. Block A, B and C were treated with Oilzapper plus nutrient, while block D was maintained as control which was treated only with nutrients.

At zero time (just before initiation of bioremediation), the oily sludge (TPH) contamination in soil of block A was 31.10%. With application of Oilzapper plus nutrient, the oily sludge (TPH) contamination in soil reduced from 31.10% to 6.64% in two months which revealed 78.6% biodegradation of oily sludge in two months (Figure 5.1). In block B, the oily sludge (TPH) contamination in soil was recorded 18.23% at zero time. With application of Oilzapper plus nutrients, the oily sludge (TPH) concentration reduced from

18.23% to 6.13% after two months which accounted for 66.37% biodegradation of TPH in two months (Figure 5.1).

Similarly block C of tank dyke area treated with Oilzapper plus nutrients showed 40.73% oily sludge contamination in soil at zero time and it was reduced from 40.73% to 15.71% after two months of treatment which revealed 61.42% biodegradation of oily sludge (TPH) in two months (Figure 5.1). However, in control block D (treated with nutrient alone) of tank dyke the oily sludge concentration reduced from 40.25% to 37.82% after two months which exhibited only 6.03% biodegradation of oily sludge (Figure 5.1).

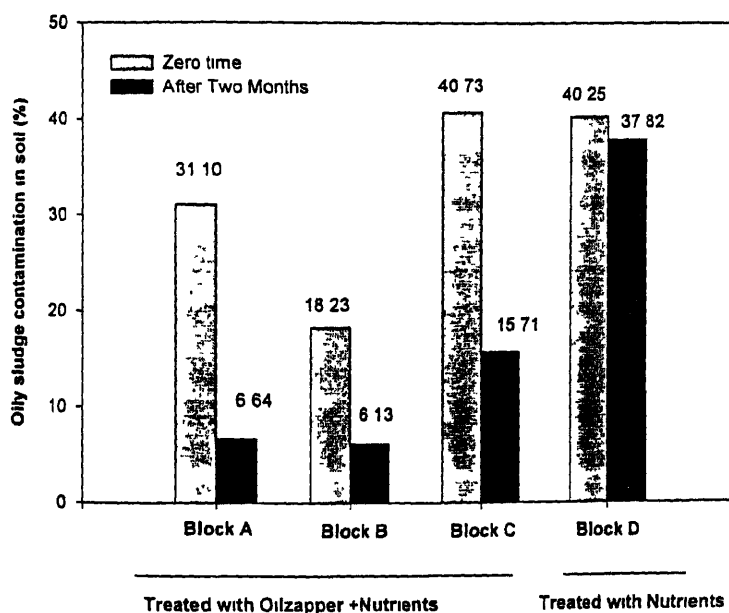


Figure 5.1 Bioremediation of Oily sludge contaminated land in tank farm area of tank A 305

Biodegradation of various fractions of oily sludge in tank dyke area of tank A305

Block A treated with Oilzapper plus nutrients revealed 78.64% biodegradation of total petroleum hydrocarbon (TPH) of oily sludge in two months, out of which biodegradation of alkane fraction was accounted for 46%, aromatic fraction 18.25% and NSO plus asphaltene fraction 14.39% in two months (Table 5.3).

Table 5.3 Biodegradation of various fractions of oily sludge in tank dyke area of tank A305

Blocks	Biodegradation (%)			
	TPH	Alkane fraction	Aromatic fraction	NSO plus asphaltene fraction
A	78.64	46	18.25	14.39
B	66.37	38	22.10	6.27
C	61.42	35.80	19.35	6.27
D	6.03	4.10	1.50	0.43

Block B of tank dyke area was treated with Oilzapper plus nutrient exhibited 66.37% biodegradation of TPH in two months. The biodegradation of alkane, aromatic and NSO plus asphaltene fraction accounted for 38%, 22.10% and 6.27% respectively in two months (Table 5.3). Similarly, block C of tank dyke area was treated with Oilzapper plus nutrients resulted in 61.42% biodegradation of TPH of which the alkane fraction was accounted for 35.80%, aromatic fraction 19.35% and NSO plus asphaltene fraction 6.27% (Table 5.3). However, in control block D, only 6.03% TPH was biodegraded in two months and biodegradation of alkane (4.1%), aromatic (1.5%) and NSO plus asphaltene (0.43%) were very less (Table 5.3).

In-situ Biodegradation of oily sludge in ETP area

In ETP area of Digboi Refinery, three sites were undertaken for biodegradation of oily sludge. In Site A, the oily sludge (solvent extractable TPH) concentration was recorded 18.46% at zero time. With application of Oilzapper plus nutrients, the sludge concentration reduced from 18.46% to 12.09% after two months which resulted in 34.5% biodegradation of sludge in two months (Figure 5.2). Site B was treated with Oilzapper plus nutrients where the oily sludge reduced from 20.09% to 10.43% in two months which showed 48% biodegradation of sludge in two months (Figure 5.2). Site C at ETP area was also treated with Oilzapper plus nutrients showed a reduction in oily sludge concentration from 21.07 to 10.17% after two months which exhibited 51.7% biodegradation of sludge (Figure 5.2).

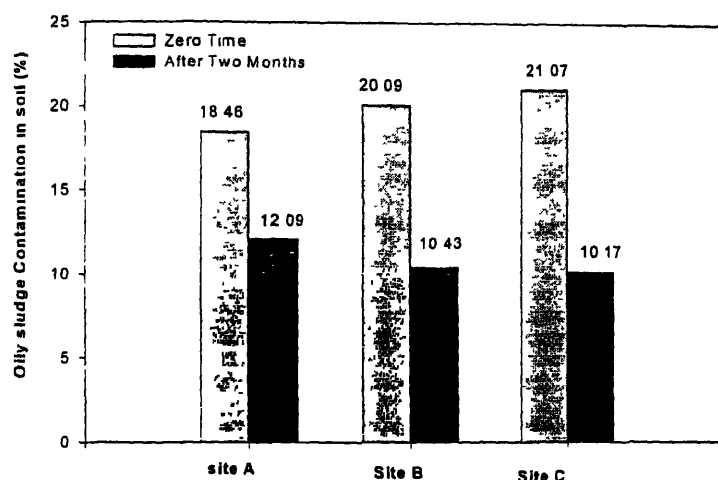


Figure 5.2 Bioremediation of oily sludge contaminated land in ETP area

Biodegradation of various fraction of sludge in ETP area

Site A in ETP area was treated with Oilzapper plus nutrient showed 34.5% biodegradation of sludge (TPH) in two months. In site A, the biodegradation of alkane fraction was 16%, aromatic fraction was 11.60% and NSO plus asphaltene fraction was 6.90% in two months (Table 5.4).

Site B in ETP area was treated with Oilzapper plus nutrient indicated 48% biodegradation of oily sludge (solvent extractable TPH) in two months of which biodegradation of alkane fraction was 26.70%, aromatic fraction 13.35% and NSO plus asphaltene fraction 7.95% in two months (Table 5.4).

Site C was also treated with Oilzapper plus nutrients, revealed 51.7% biodegradation of oily sludge (TPH) in two months. The biodegradation of alkane, aromatic, and NSO plus asphaltene fractions were 27.90%, 13.50% and 10.30% respectively in two months (Table 5.4).

Table 5.4 Biodegradation of various fractions of oily sludge in treatment sites in ETP area

Site	Biodegradation (%)			
	TPH	Alkane fraction	Aromatic fraction	NSO plus asphaltene fraction
A	34.5	16.00	11.60	6.90
B	48.0	26.70	13.35	7.95
C	51.7	27.90	13.50	10.30

In-situ Bioremediation of oily sludge in sludge drying pit 6

Sludge drying pit 6 (in ETP area) was undertaken for bioremediation of oily sludge. At zero time, the solvent extractable TPH content in sludge was 51.14%. With application of Oilzapper plus nutrients in sludge pit, the TPH content in sludge reduced from 51.14% to 28.86% in two months revealed 43.56% bioremediation of TPH content in sludge (Figure 5.3).

Estimation of biodegradation of various fractions of TPH in sludge drying pit 6 revealed 22.65% biodegradation of alkane fraction, 12.80% biodegradation of aromatic fraction and 8.11% biodegradation of NSO plus asphaltene fractions in two months.

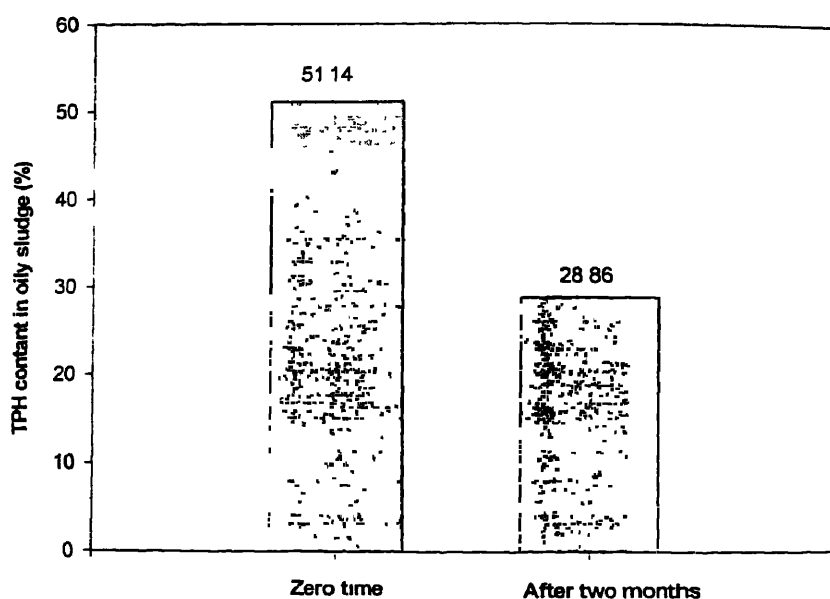
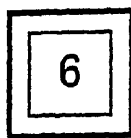


Figure 5.3 Bioremediation of oily sludge in sludge drying pit 6

Summary

- At Digboi refinery, tank farm area of tank A305, sludge drying pit 6 and old sludge dumped site were undertaken for bioremediation of oily sludge.
- In tank farm area, the blocks treated with Oilzapper plus nutrients resulted in 61.42% to 78.64% biodegradation of oily sludge (total petroleum hydrocarbon) in two months as compared to 6.03% biodegradation of oily sludge in untreated block (treated with nutrients alone).

- Oily sludge in sludge drying pit 6 was treated with Oilzapper plus nutrients exhibited that oil content in sludge reduced from 51.14% to 28.86% in two months resulted in 43.56% biodegradation of total petroleum hydrocarbon (oily sludge) in two months.
- The sludge dumped site A, B, and C in ETP area treated with Oilzapper plus nutrients revealed 34.5%, 48% and 51.7% *in-situ* biodegradation of TPH at sites A, B and C respectively in two months.



Field trial of oilzapper at Oil India Limited, Duliajan

Methods

Mass scale production of Oilzapper and its application in sludge pits and at oil contaminated water

"Oilzapper" (sludge degrading bacterial system) was grown in a fermenter on minimal salt medium. After 16 hours of growth cultures were harvested and mixed with a carrier material. The carrier based Oilzapper (containing 10^{10} cells/g carrier material) was filled in sterilized polythene bags and. Sealed culture packets were kept in dark at 25°C for 7 days for curing. Cured culture packets were transported to Oil India Ltd., Duliajan and applied in experimental sludge treatment site.

Composition of sludge collected from sludge pits in tank farm area

Oily sludge collected from sludge pits of tanks was analysed before initiation of bioremediation work. The solvent extractable total petroleum hydrocarbon from sludge was extracted using hexane, methylene chloride and chloroform. After evaporation of solvents, the amount of TPH recovered from 10 g of sludge was quantified. The TPH recovered from sludge was further fractionated into alkane, aromatic, asphaltene and NSO (nitrogen, sulphur, oxygen containing compounds) fractions with silica gel column. After TPH extraction, the residual sludge (1 g) was heated at 600°C for five hours. After cooling, the amount of sediments/ash recovered from residual sludge was quantified by gravimetrically.

Composition of TPH recovered from oily sludge

The TPH recovered from oily sludge was fractionated into alkane, aromatic asphaltene and NSO (nitrogen, sulphur and oxygen containing compound) with a silica-gel column. The TPH was first dissolved in n-pentane and separated into soluble and insoluble fractions (asphaltene). The soluble fraction was loaded on silica-gel column and eluted with different solvents. The alkane fraction was eluted with hexane, followed by the aromatic fraction that was eluted with benzene. The NSO fraction was eluted with chloroform and methanol. Alkane and aromatic fractions were concentrated by evaporation of solvents and then 0.2 µl of each was analyzed by gas chromatography (GLC Hewlett Packard, 5890

Series II) to identify all the compounds present in the alkane and aromatic fractions. In the alkane fraction, various alkane compounds were identified by matching the retention time with authentic standards. Simultaneously, the identification of each alkane compound was also confirmed by spiking the alkane fraction. A known amount of each standard alkane compound was added to the alkane fraction and injected into GC (co-injection) and the peak area of each alkane compound increased as a result. Similarly, the polycyclic aromatic hydrocarbon compounds were identified by matching the retention time with an authentic standard and confirmed by spiking the sample with authentic standards.

Selection of site for bioremediation of oily sludge

Tank farm area

At Oil India Ltd., Duliajan, the site for bioremediation of oily sludge was selected in sludge pits in tank farm area of tank No. 1,5 & 6. Three sludge pits which were situated in tank dyke area of tanks were selected for bioremediation of oily sludge. Each pit was containing approximately 150-200 tonnes sludge. In each pit, approximately 80-100 kg Oilzapper was applied on oily sludge and nutrients were also sprayed on sludge along with Oilzapper.

After applying Oilzapper and nutrient on sludge in pits, approximately 100-150 kg soil in each pit was applied on sludge for providing air pockets in sludge for the bacteria. After applying soil in the pits, the sludge in all three pits was mixed with Oilzapper and soil by tilling. The zero time sampling (10 samples from each pit) from each pit was done just after 1st tilling. The tilling in all three pits were carried out at periodic intervals. The sampling (10 samples from each pit) from all three pits were also carried out after two months of initiation of bioremediation work. The samples were transported to the laboratory at TERI, New Delhi for analysis. Oily sludge was heterogeneously distributed in the samples collected from sludge pits at zero time and after 60 days and therefore, the sample was powdered by soil pulverizer, and then solvent extractable total petroleum hydrocarbon (TPH) from 10 g sample was extracted with hexane methylene chloride and chloroform (80 ml each) consecutively. After extraction, all three extracts obtained by hexane, methylene chloride and chloroform were pooled and dried at room temperature by evaporation of solvent under gentle nitrogen stream in a fumehood. After solvent evaporation, the amount of TPH recovered from 10 g sample collected from pits at zero time and after two months were determined by simple gravimetry. The difference in

TPH concentration of 10 g sample of zero time and after two months revealed the amount of sludge (TPH) biodegraded in sludge pits.

Biodegradation of various fractions of oily sludge in sludge pits

The TPH recovered from 10 tank sludge collected at zero time and different intervals was further fractionated into alkane, aromatic, NSO and asphaltene fractions on silica gel column as above. The concentration of these fractions was estimated by gravimetry.

Oil Collection Centre(OCS), Joypur

Water (formation water) pit near Joypur OCS was selected for bioremediation. Pit size was approximately 100m x 30m. Water in pit was contaminated with crude oil. A thick layer of crude oil (approximately 4 inch thick) was floating on surface water.

In the month of February 2000, a heavy dose of Oilzapper (150 kg) and nutrients (100 litres) was applied in one-third portion of pit. Oilzapper and nutrients were mixed with crude oil floating on surface water by using big bamboo sticks. A boat was used to apply Oilzapper and nutrients in the water pit. At zero time (just before application of Oilzapper and nutrients), ten samples of crude oil were collected from the pit where Oilzapper was applied (one third portion of pit). At the opposite end of this pit, a control was maintained. Crude oil samples (10 samples) were also collected from control. Samples were also collected in the month of April 2000 (after two months of application of water). The composition of crude oil collected from water pit at zero time and after two months of treatment was estimated and thus biodegradation of various fractions of crude oil was quantified.

Biodegradation of various fractions of crude oil

Crude oil samples collected from water pit at Joypur OCS at zero time and after two months of application of Oilzapper was fractionated into alkane, aromatic, NSO (Nitrogen, sulphur, oxygen containing compound) and asphaltene as described above. The relative concentration of these fractions in crude oil collected was estimated.

Results

Composition of oily sludge of crude tank No.1

Before initiation of bioremediation in sludge pit of tank No.1, the composition of sludge was estimated. The composition of oily sludge shows 57% solvent extractable total petroleum hydrocarbon (TPH), 15.8% water content, 20.2% sediment/ash and 7% organic matter (Table 6.1).

Table 6.1 Composition of oily sludge of sludge pit 1

Composition	Results
Total petroleum hydrocarbon (solvent extractable)	57.0
Water content	15.8
Organic matter	7.0
Sediments/ash	20.2

The TPH was further fractionated into various fractions showed 49% alkane fraction, 28% aromatic fraction, 9% NSO fraction and 14% asphaltene fraction (Table 6.2).

Table 6.2 Composition of TPH of oily sludge pit 1

Fraction	Percent (%)
Alkane fraction	49%
Aromatic fraction	28%
NSO fraction	9%
Asphaltene fraction	14%

The composition of oily sludge of sludge pit 5 was estimated before initiation of bioremediation work. Oily sludge of pit 5 contained 58% solvent extractable TPH, 22% water content, 9% organic matter (wax and other organic waste) and 11% sediment, ash and inorganic waste (Table 6.3). The TPH extracted from oily sludge was further fractionated into different fractions. The composition of TPH revealed 47% alkane fraction, 34% aromatic fraction, 8% NSO fraction and 11% asphaltene fraction (Table 6.4).

Table 6.3 Composition of oily sludge of sludge pit 5

Composition	Result
Solvent extractable total petroleum hydrocarbon	58%
Water content	22%
Organic matter	9%
Sediment/ash content	11%

Table 6.4 Composition of TPH of oily sludge of sludge pit 5

Composition	Result
Alkane fraction	47%
Aromatic fraction	34%
NSO fraction	8%
Asphaltene	11%

The composition of oily sludge of pit 6 was also estimated just before bioremediation of oily sludge. Oily sludge of pit 6 contained 45% solvent extractable TPH, 17% water content, 12% organic matter and 26% sediments/ash content (Table 6.5). The TPH extracted from oily sludge was further fractionated into different fractions. The composition of TPH revealed 43% alkane fraction, 30% aromatic fraction, 9% NSO fraction and 18% asphaltene fraction (Table 6.6).

Table 6.5 Composition of oily sludge of sludge pit 6

Composition	Result
Solvent extractable total petroleum hydrocarbon	45%
Water content	17%
Organic matter	12%
Sediment/ash content	26%

Table 6.6 Composition of TPH of oily sludge of sludge pit 6

Composition	Result
Alkane fraction	43%
Aromatic fraction	30%
NSO fraction	9%
Asphaltene	18%

Bioremediation of oily sludge in sludge pits

Bioremediation of oily sludge in sludge pits 1, 5 and 6 situated in tank dykes of tank 1, 5 and 6 was initiated in the month of November 1999. Heavy dose of Oilzapper and nutrients was applied on oily sludge in all three sludge pits. In sludge pit 1 at zero time (just after application of Oilzapper), the oil content in sludge (solvent extractable TPH) was 41.06%. With application of Oilzapper and nutrients, the oil content in sludge reduced from 41.06% to 8.91% after four months which resulted in 78.3% biodegradation of oily sludge (Figure 6.1). In

sludge pit 5, the oil content in sludge was recorded 37.10% at zero time. Treatment of oily sludge with Oilzapper and nutrients, the oil content in sludge reduced from 37.10% to 18.20% after four months which revealed 50.9% biodegradation of oily sludge. Similarly, with application of Oilzapper and nutrients, the oil content in oily sludge of sludge pit 6 reduced from 36.4% to 12.08% in four months which resulted in 66.8% biodegradation of oily sludge (Figure 6.1).

Biodegradation of various fractions of oily sludge

In sludge pit 1, out of 78.3% biodegradation of oil content in four months, the biodegradation of alkane fraction was accounted for 45%, aromatic fraction 24%, NSO fraction 6% and asphaltene fraction 3.3% (Table 6.11).

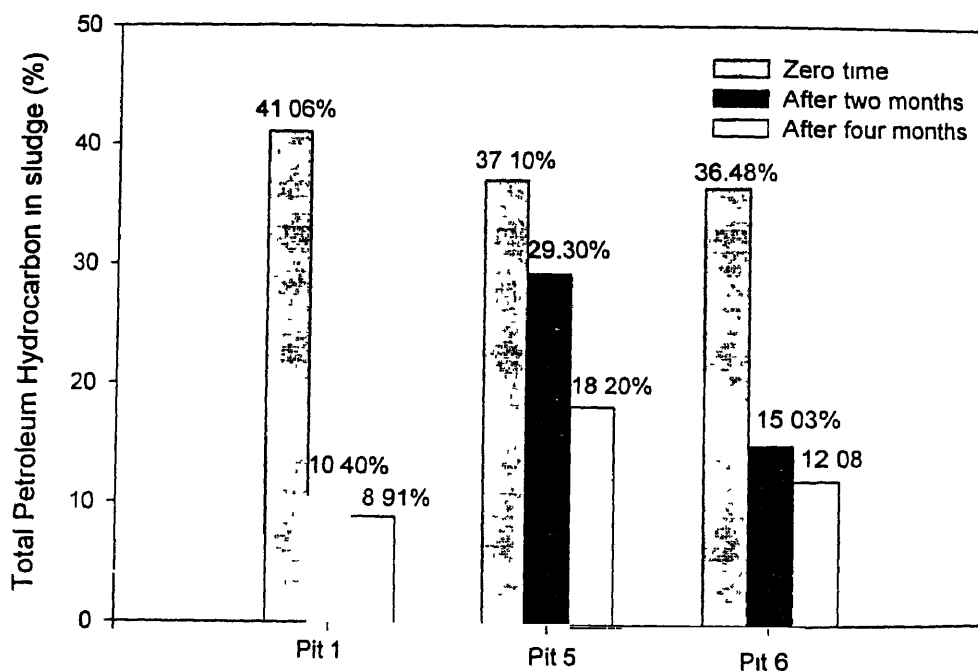


Figure 6.1 Biodegradation of oily sludge in sludge pits

Table 6.11 Biodegradation of various fraction of oily sludge in four months

Oily sludge fraction	Biodegradation (%)		
	Pit 1	Pit 5	Pit 6
Total petroleum hydrocarbon	78.3	50.9	66.8
Alkane fraction	45.0	24.5	34.5
Aromatic fraction	24.0	17.8	23.8
NSO fraction	6.0	4.4	4.6
Asphaltene fraction	3.3	4.2	3.0

In sludge pit 5, out of 50.9% biodegradation of oil content in four months, the alkane fraction was 24.5%, aromatic fraction 17.8%, NSO fraction 4.4% and asphaltene fraction 4.2% (Table 6.11).

In sludge pit 6, total 66.8% of oily sludge was biodegraded in four months. The biodegradation of alkane, aromatic, NSO and asphaltene fractions were accounted for 35.4%, 23.8%, 4.6% and 3% respectively

Bioremediation of crude oil contaminated water near Joypur OCS

Water (formation) pit contaminated with crude oil was selected for bioremediation. In one third of this pit, Oilzapper and nutrients were applied on crude oil layer floating on water. With application of Oilzapper and nutrients, the crude oil was completely biodegraded in one third portion of water pit. The GC fingerprinting of biodegradation of alkane and aromatic fraction of crude oil is shown in Figure 11 and 12 respectively.

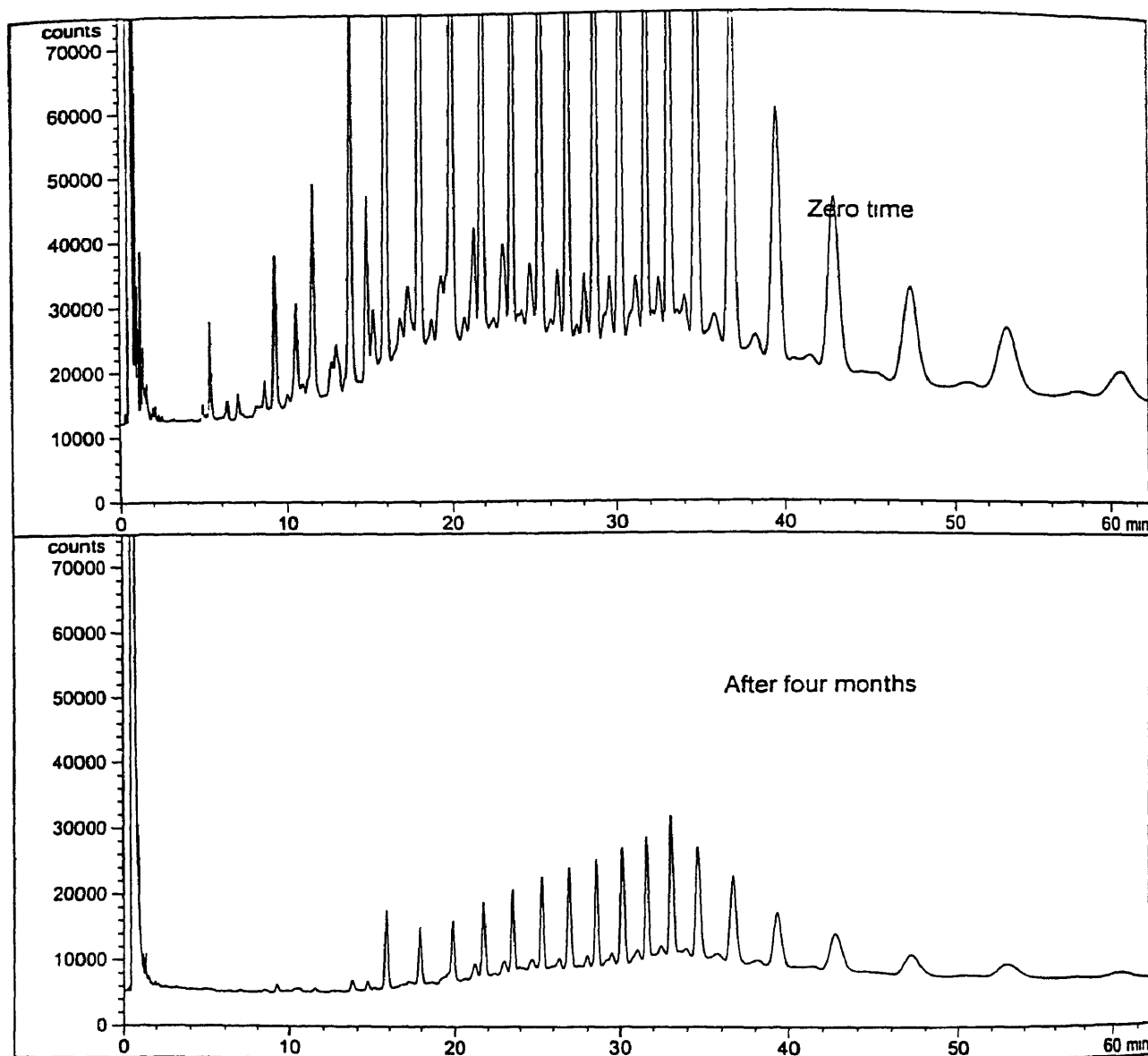


Figure 6.2 GC fingerprinting of alkane fraction of crude oil collected from water pit near Joypur OCS

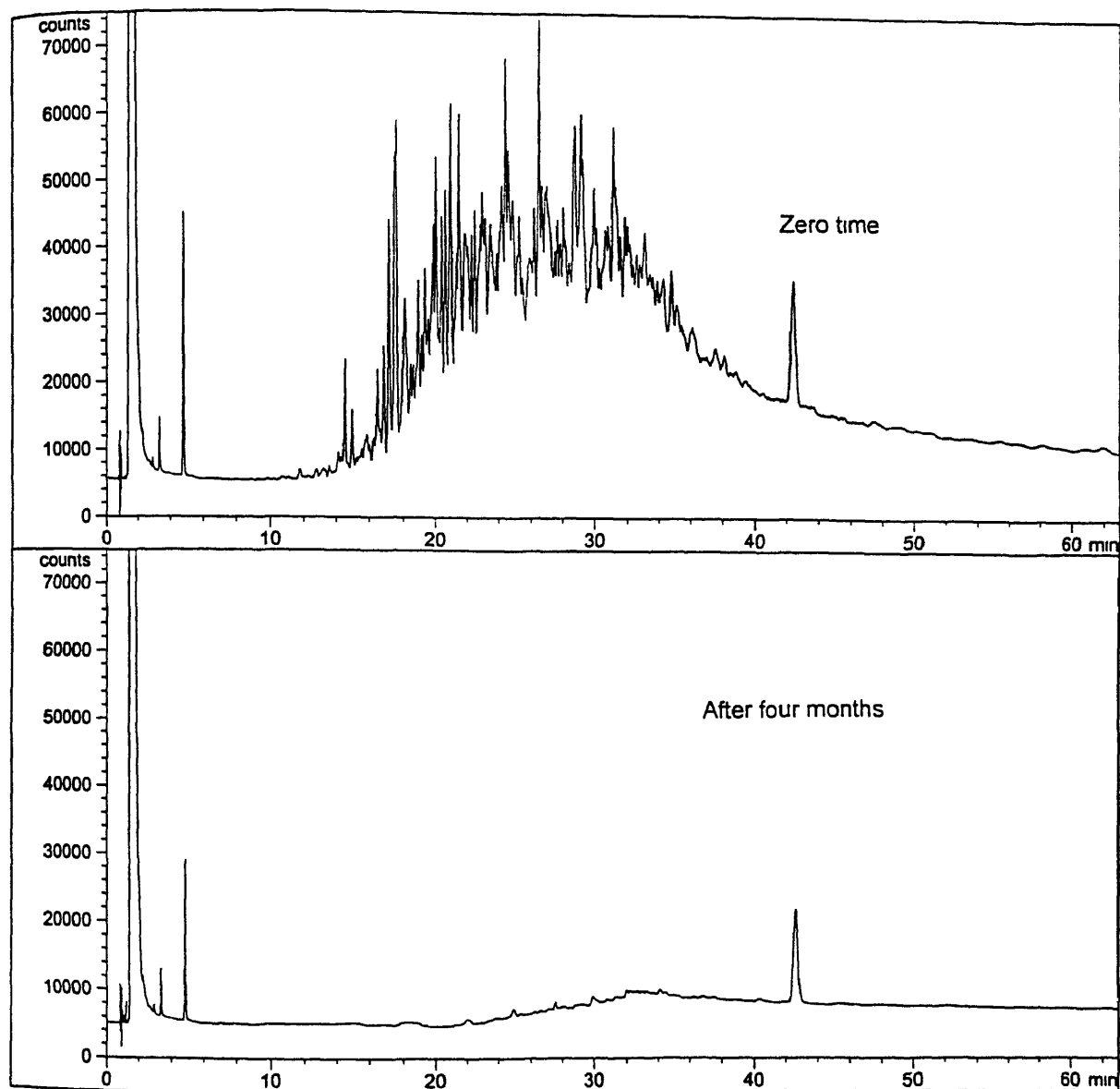


Figure 6.3 GC fingerprinting of aromatic fraction of crude oil collected from water pit near Joypur
OCS

Summary

- Bioremediation of oily sludge in sludge pit 1, 5 and 6 were initiated in the month of November 1999. With encouraging results on bioremediation of oily sludge, two more sludge pits (pit 2 and 4) were undertaken for bioremediation of oily sludge.
- In sludge pit 1, total 78.3% oily sludge has been biodegraded by Oilzapper in four months.
- In sludge pit 5, with application of Oilzapper, 50.9% oily sludge has been biodegraded in four months.
- In sludge pit 6, 66.8% oily sludge has been biodegraded in four months by applying Oilzapper on oily sludge.
- At Bowser unloading site near OCS 3, soil was contaminated with crude oil spill. At this site, crude oil contaminated soil was treated with Oilzapper and nutrients. Treatment of this site with Oilzapper and nutrients resulted in the reduction of crude oil contamination in soil from 38.47% to 13.50% in four months.
- At Joypur OCS, water pit contaminated with crude oil was also undertaken for bioremediation. Oilzapper and nutrients was applied in one-third portion of this pit which resulted in complete biodegradation of crude oil floating on surface water of the pit.



Field trial of oilzapper at Guwahati refinery

Methods

Selection of site for field trial of Oilzapper

Field trial of Oilzapper for treatment of Oily sludge at Guwahati Refinery was initiated in the month of 1999. At Guwahati Refinery, approximately 30m x 20m area was selected for bioremediation of oily sludge. This site was situated towards the upper side of two sludge pits (near flair area). Selected sites got contaminated with oily sludge while dumping the sludge in sludge pits.

A soil boundary wall (18' height x 6" width) was prepared around selected site. Approximately 80 MT oily sludge was taken out from the pit and applied in selected site (within boundary wall). After sludge dumping in bioremediation site, Oilzapper was applied along with 100 litre nutrients on oily sludge. Just after application of Oilzapper and nutrient on sludge, manual tilling was carried out to mix oily sludge and Oilzapper uniformly.

Analysis of soil of sludge treatment plot

Selected site was contaminated with oily sludge (sludge mixed with cock dust). At zero time (before initiation of bioremediation), the soil samples were collected from different depths of sludge treatment plot. Zero time soil samples were analysed for physico-chemical characterisation of soil. Soil samples would be collected after completion of the study also and would be analysed as described above.

Oilzapper production and its application at Guwahati Refinery

Oilzapper (an oily sludge degrading bacterial strain) culture was produced in a bioreactor at TERI laboratory at New Delhi. After sufficient growth of Oilzapper in bioreactor, the bacterial cells (Oilzapper) were mixed with a carrier material. The carrier based Oilzapper (containing 10^{10} cells/g carrier material) was filled in sterilized poly bags. Poly bags containing Oilzapper were sealed and kept in dark room at 25°C for seven days for curing. Cured Oilzapper containing poly bags were transported to Indian Oil Corporation Ltd., Guwahati Refinery for treatment of oily sludge.

Since oily sludge was mixed with cock fine and oil content was also high in oily sludge, heavy dose of Oilzapper was applied on oily sludge (1 kg Oilzapper/400 kg sludge). After application of Oilzapper on sludge, tilling of sludge was carried out for proper mixing of Oilzapper in sludge. Oilzapper also required nutrients to biodegrade oily sludge. Therefore, a mixture of nutrients was also sprayed on oily sludge. A control was also maintained where only mixture of nutrient was sprayed on oily sludge.

Characterisation of oily sludge biodegraded at Guwahati refinery

Oily sludge (80 MT sludge) that was biodegraded at Guwahati Refinery was characterised before initiation of bioremediation of sludge.

Water content in sludge was estimated by sodium sulphate. After removal of water from sludge, oil content (TPH) in sludge was estimated. Oil content was extracted from oily sludge using hexane, methylene chloride and chloroform. After evaporation of solvents, the amount of oil recovered from 10g oily sludge was quantified. The oil (TPH) that was extracted from oily sludge was further fractionated into alkane, aromatic, asphaltane and NSO (nitrogen, oxygen, sulphur containing compound) with silicagel column. The ash content of oily sludge was estimated after extraction of TPH from sludge. A known amount of residual oily sludge was taken in crucible and heated at 600°C for 5 hours. After cooling, the amount of ash recovered from 10g sludge was quantified by gravimetry.

Monitoring the rate of biodegradation of oily sludge at Guwahati Refinery

At zero time (just after application of Oilzapper and nutrients on oily sludge), oily sludge samples were collected from 20 points from bioremediation plot. Sludge samples (20) were also collected from bioremediation plot after two months of application of Oilzapper and nutrients on sludge. Sludge samples were transported to the TERI laboratory at New Delhi for analysis. Total petroleum hydrocarbon (TPH) from 10g sludge samples collected at zero time and after two months were extracted with hexane, methylene chloride and chloroform (100 ml each) consecutively (93-95% extraction efficiency). After extraction, all the three extracts (obtained by hexane, methylene chloride and chloroform) were pooled and dried at room temperature by evaporation of solvents under gentle nitrogen stream in fumehood. After solvent evaporation, the amount of TPH recovered from 10g sludge was determined by simple gravimetry method. The difference in oil content in sludge at zero time and after

two months revealed the amount of sludge (TPH) biodegraded by Oilzapper in two months.

Composition of TPH recovered from oily sludge of Guwahati refinery

The TPH recovered from oily sludge at zero time and after 2 months were fractionated into alkane, aromatic asphaltane and NSO (nitrogen, oxygen, and sulphur containing compound) with a silica-gel column. The TPH was first dissolved in n-pentane and separated into soluble and insoluble fractions (asphaltenes). The soluble fraction was loaded on silica-gel column and eluted with different solvents. The alkane fraction was eluted with hexane, followed by the aromatic fraction that was eluted with benzene. The NSO fraction was eluted with chloroform and methanol. Alkane and aromatic fractions were concentrated by evaporation of solvents and then 0.2 µl of each was analyzed by gas chromatography (GC Hewlett Packard, 5890 Series II) to identify all the compounds present in the alkane and aromatic fractions. In the alkane fraction, various alkane compounds were identified by matching the retention time with authentic standards. Simultaneously, the identification of each alkane compound was also confirmed by spiking the alkane fraction. A known amount of each standard alkane compound was added to the alkane fraction and injected into GC (co-injection) and the peak area of each alkane compound increased as a result. Similarly, the polycyclic aromatic hydrocarbon compounds were identified by matching the retention time with an authentic standard and confirmed by spiking the sample with authentic standards.

Results

Physico-chemical properties of soil of bioremediation site

Before initiation of bioremediation of oily sludge at Guwahati Refinery, soil of 25 cm horizon (surface to 25 cm depth), 50 cm horizon (25 to 50 cm depth), 75 cm horizon (50 to 75 cm depth) and 100 cm horizon (75 to 100 cm depth) were collected and analysed in the laboratory and results are shown in Table 1. Zero time soil analysis of site showed that texture of soil was black due to sludge contamination. Fine gravels were present in soil. At zero time, the water holding capacity of soil was less (49%) as compared to normal agriculture land. The pH of soil was also less (pH 5.5).

The organic carbon in soil was high (3.9%) as compared to normal agriculture land. The available forms of phosphorus in soil was less while potassium was comparable to normal agriculture land (Table 7.1). The soil of

bioremediation site will be tested again after completion of bioremediation of oily sludge.

Table 7.1 Physical and chemical properties of soil of 25 cm horizon of sludge treatment plot at zero time

Test	Results
Texture	Black due to sludge
Soil bulk density	0.98g/cm ³
Water holding capacity	49%
PH	5.5
Organic carbon	3.9%
Total nitrogen	0.05%
Available phosphorus	38 ppm
Potassium	180 ppm

Composition of oily sludge biodegraded at Guwahati refinery

The composition of oily sludge which was biodegraded by Oilzapper was estimated. Cock dust was mixed with oily sludge. Therefore, sludge was looking black with fine granules. Oily sludge contained 18% (w/w) water. Total petroleum hydrocarbon (TPH) on extraction shows solvent extractable TPH 55.93% and sediment/ash content 26.07%. The solvent extracted TPH was further fractionated into various fractions showed 58% alkane fraction, 38% aromatic fraction, 1% NSO and 3% asphaltane fraction as shown in Table 7.2.

Table 7.2 Characterisation of oily sludge

Constituent of oily sludge	Composition (%)
Water	18
Solvent extractable TPH	55.93
Sediment/ash content	26.07
Constituent of TPH extraction from oily sludge	
Alkane fraction	58
Aromatic fraction	38
NSO fraction	1
Asphaltane fraction	3

Note: Values are mean of 10 samples

In-situ Bioremediation of oily sludge at Guwahati refinery

Oily sludge contaminated site (approximately 30 m x 20 m area) was undertaken for bioremediation of oily sludge. Since the site was contaminated with oily sludge but the sludge which was lying at site was less, therefore, approximately 80 metric tonnes of additional oily sludge (in addition to the sludge already present) was applied at site for biodegradation of sludge.

At zero time, the oil content (TPH) in sludge was 55.93%. With application of Oilzapper and nutrients, the oil content in sludge reduced from 55.93% to 15.63% after two months of application of Oilzapper which resulted in 72% biodegradation of TPH in oily sludge (Figure 7.1). However, in control where only nutrients were sprayed, showed 56.21% oil content in sludge at zero time and 50.40% oil content in sludge after two months which exhibited only 10.3% biodegradation of oily sludge in two months (Figure 7.1).

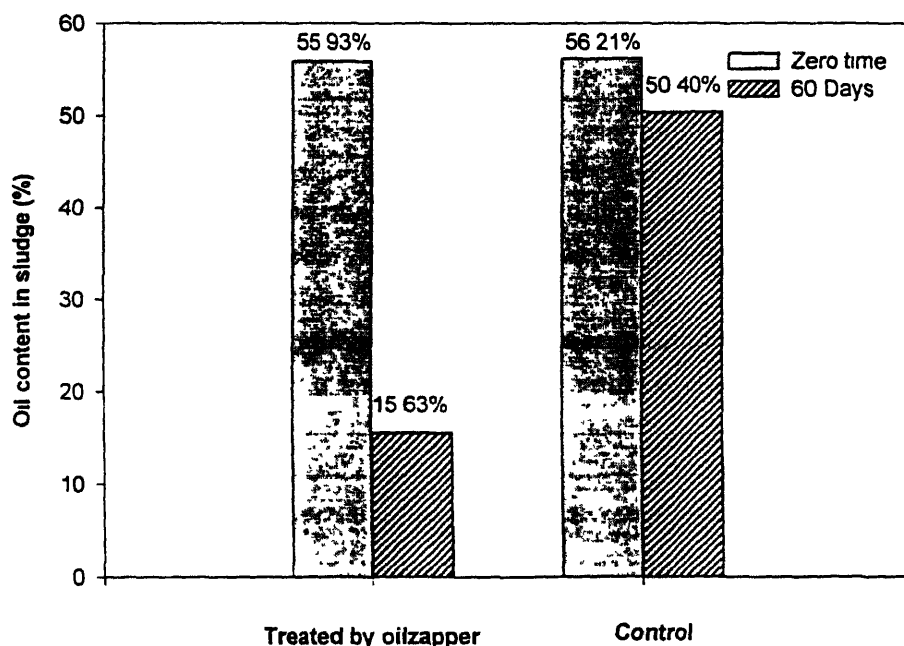


Figure 7.1 Biodegradation of oily sludge at Guwahati Refinery

In-situ Biodegradation of various fractions of oily sludge

Biodegradation of various fractions of oily sludge at bioremediation site was estimated. Sludge which was treated with Oilzapper plus nutrient showed 72% biodegradation of oil content of sludge in two months. Out of 72% biodegradation of oil, the biodegradation of alkane fraction was accounted for 38%, aromatic fraction was 32% and rest 2% was accounted for biodegradation of NSO and asphaltane fraction (Table 7.3). In control only 10.3% oil content was biodegraded by natural occurring bacteria at site in two months of which biodegradation of alkane fraction was 6.1%, aromatic fraction 3.8% and only 0.4% was of NSO and asphaltane fraction (Table 7.3).

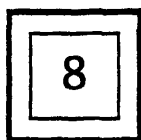
Table 7.3 Biodegradation of various fractions of oily sludge

Fraction	Biodegradation (%)	
	Sludge treated with Oilzapper + nutrients	Control (sludge treated with nutrients)
Total petroleum hydrocarbon	72	10.3
Alkane fraction	38	6.1
Aromatic fraction	32	3.9
NSO + Asphaltane	2	0.4

Summary

- Bioremediation of oily sludge at Guwahati Refinery was initiated in the month of February 2000. Oily sludge biodegraded at Guwahati Refinery was mixed with cock fine.
- Approximately 30m x 20m plot area contaminated with oily sludge were selected for bioremediation. In this plot, approximately 80 MT sludge was added in addition to the already present sludge.
- Heavy dose of Oilzapper and nutrients was applied on sludge.
- At zero time, the oil content in sludge was 55.93%. With application of Oilzapper and nutrients, the oil content in sludge reduced from 55.93% to 15.63% after two months, which revealed 72% biodegradation in two month. However, in control (sludge treated only with nutrients), the oil content in sludge was 56.2% at zero time and 50.40% after two months which showed only 10.3% biodegradation of total petroleum hydrocarbon.
- Sludge which was biodegraded by Oilzapper and nutrients revealed that most of the alkane and aromatic fractions of oily sludge have been biodegraded in two months as shown in Figure 3 and 4 respectively.

However, in untreated sludge, the biodegradation of alkane and aromatic fractions of oily sludge was very less as shown in Figure 5 and 6 respectively.



Field trial of oilzapper at HPCL refinery, Visakhapatnam

Methods

Selection of site for field trial of Oilzapper for mediation of oily sludge at HPCL refinery

Oily sludge bioremediation site was selected in new tank farm area situated outside the refinery campus. A pit (size 30 m x 20 m) which was having approximately 350 metric tonnes of oily sludge was selected for bioremediation. Initially in the month of May 2000, first dose of Oilzapper (200 kg) and 200 litres of nutrients were applied on oily sludge. Tilling of sludge was carried out using JCB for uniform mixing of Oilzapper bacterial cells in sludge (Figure 1). Oily sludge samples collected at time zero, after two, four and six months from the treatment pit were transported to TERI laboratory at New Delhi for analysis.

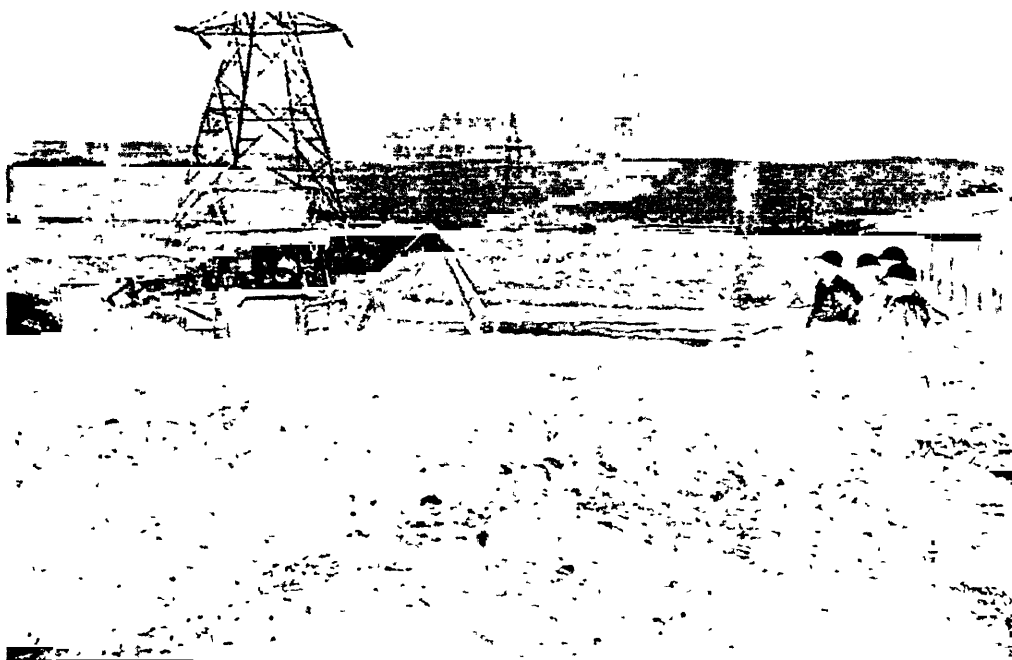


Figure 8.1 Tilling of sludge after application of Oilzapper on sludge

Oilzapper application on oily sludge at HPCL Refinery, Visakhapatnam

Oilzapper (an oily sludge degrading bacterial system) was produced in a bioreactor at TERI laboratory, New Delhi. After optimum growth of Oilzapper, bacterial cells were mixed with a carrier material. The carrier based Oilzapper (containing 10^8 cells/g carrier material) was filled in sterile polybags and transported to HPCL Refinery, Visakhapatnam for treatment of oily sludge. In the month of May 2000, first dose of Oilzapper (200 kg Oilzapper) and 200 litres of nutrients were applied on oily sludge. In the month of July 2000, second dose of Oilzapper (150 kg Oilzapper) and nutrients (200 litres) were applied on oily sludge. In the month of September 2000, third dose of Oilzapper (50 kg) and nutrients (100 litres) were applied and tilling of sludge was carried at each time of application of Oilzapper by JCB for proper mixing of bacterial cells in sludge.

Monitoring the rate of biodegradation of oily sludge

Sludge samples collected from the treatment pit at zero time, after two, four and six months of initiation of bioremediation of oily sludge were transported to TERI laboratory, New Delhi for analysis.

Total petroleum hydrocarbon (TPH) from 10 g sludge samples collected at zero time, after two, four and six months were extracted with hexane, methylene chloride and chloroform (80 ml each) consequently (93-95% extraction efficiency). After extraction, all the three extracts (obtained by hexane, methylene chloride and chloroform) were pooled and dried at room temperature by evaporation of solvents under gentle nitrogen stream in fumehood. After solvent evaporation, the amount of TPH recovered from 10g sludge was determined by simple gravimetry method.

In-situ Degradation of various fractions of oily sludge

The TPH recovered from oily sludge at zero time and after two, four and six months were fractionated into alkane, aromatic asphaltene and NSO (nitrogen, oxygen, and sulphur containing compound) with a silica-gel column. The TPH was first dissolved in n-pentane and separated into soluble and insoluble fractions (asphaltene). The soluble fraction was loaded on silica-gel column and eluted with different solvents. The alkane fraction was eluted with hexane, followed by the aromatic fraction that was eluted with benzene. The NSO fraction was eluted with chloroform and methanol. Alkane and aromatic fractions were concentrated by evaporation of solvents and then 0.2 μ l of each was analyzed by gas chromatography (GC –FID Hewlett Packard, 5890 Series

II) to identify all the compounds present in the alkane and aromatic fractions. In the alkane fraction, various alkane compounds were identified by matching the retention time with authentic standards. Simultaneously, the identification of each alkane compound was also confirmed by spiking the alkane fraction. A known amount of each standard alkane compound was added to the alkane fraction and injected into GC (co-injection) and the peak area of each alkane compound increased as a result. Similarly, the polycyclic aromatic hydrocarbon compounds were identified by matching the retention time with an authentic standard and confirmed by spiking the sample with authentic standards.

Characterisation of oily sludge

Oily sludge (350 MT sludge) that was biodegraded at HPCL Refinery was characterised before initiation of bioremediation of sludge. Water content in sludge was estimated by sodium sulphate. After removal of water from sludge, oil content (TPH) in sludge was estimated. Oil content was extracted from oily sludge using hexane, methylene chloride and chloroform. After evaporation of solvents, the amount of oil recovered from 10g oily sludge was quantified. The oil (TPH) that was extracted from oily sludge was further fractionated into alkane, aromatic, asphaltene and NSO (nitrogen, oxygen, sulphur containing compound) with silica gel column. The ash content of oily sludge was estimated after extraction of TPH from sludge. A known amount of residual oily sludge was taken in crucible and heated at 600°C for 5 hours. After cooling, the amount of ash recovered from 10g sludge was quantified by gravimetry.

Analysis of soil of sludge treatment plot

Soil samples were collected from sludge treatment pit at zero time and six months. Soil samples collected at zero time and after six months of initiation of treatment were analysed for physico-chemical characterisation of soil.

Analysis of trace metals in soil contaminated with sludge

Various trace metals were analysed from soil samples collected at zero time and after six months. Oily sludge containing soil samples were digested in nitric acid. A known weight of soil was taken in clean 100 ml beaker. A 15 ml concentrated nitric acid was added to beaker and the beaker was covered with a watch glass and heated at 140°C on a hot plate, in a fume hood, until most of the acid was evaporated. The step was repeated thrice in order to dissolve the metallic components. The solution was then filtered in another beaker through

0.45 micron Whatman filter paper number 42 and the insoluble residues on the filter paper was rinsed with 10% nitric acid. The residue was then discarded and beaker was covered with a watch glass and heated at 140°C until complete evaporation of nitric acid. Now the beaker was heated at 400°C until barely dried and white ash appeared. The sample was not allowed to bake and temperature was maintained at 400°C for six hours. The bottom of the watch glass was carefully rinsed into the beaker using 10% nitric acid. The sides of the beaker was also rinsed and solution was evaporated to dryness at 140°C. The filter paper blank and nitric acid blank were also prepared similarly. Each beaker was then cooled and the residue was dissolved in 1 ml of concentrated nitric acid. The clear solution was then quantitatively transferred into 50 ml volumetric flask and volume was made up by using 10% nitric acid. The extract was initially analysed by flame (air-acetylene) AAS using standard method. Some metals such as Se, and As, were analysed by AAS equipped with hydrate system.

Results

Physico-chemical properties of soil of soil of bioremediation site

Zero time and after six months soil analysis showed that texture of soil remained unchanged. At zero time, the water holding capacity of soil was 45.18%, which increased to 57.9% in six months. pH of soil at sludge treatment site remained unchanged. However soil bulk density of sludge treatment site increased from 1.15 g/cm³ to 1.30g/cm³, which is comparable to agriculture soil. Organic carbon in soil at sludge treatment site increased from 1.90% to 2.60% in six months which was due to oily sludge contamination in soil. Available phosphorus in soil also increased 4.6 ppm to 18.7 ppm in six months. Potassium in soil at treatment site increased from 65 ppm to 72 ppm in six months. Similarly total nitrogen in soil also increased significantly in six months. (Table 8.1).

Selected heavy metals were analysed in soil samples contained oily sludge collected from TERI bioremediation site at zero time and after six months. Concentration of all the heavy metals in soil reduced significantly in six months. Among nine heavy metals analysed the maximum reduction was of Fe Concentration (Table 8.2). Concentration of all the heavy metals analysed in soil samples were within the permissible limits.

Table 8.1 Physical properties of soil of TERI bioremediation site

Test	Zero time	After six months
Total petroleum hydrocarbon contamination in soil	--	6.7%
pH	7.35	7.40
Soil texture	Red with gravel	Red colour
Water holding capacity of soil	45.18%	57.9%
Soil bulk density	1.15 g/cm ³	1.30 g/cm ³
Organic carbon in soil	1.90%	2.60%
Available phosphorus in soil	4.6 ppm	18.7 ppm
Potassium in soil	65 ppm	72 ppm
Total nitrogen in soil	0.08%	0.092%

Table 8.2 Heavy metal concentration (ppm) in soil samples of TERI bioremediation site

Heavy metal	Concentration (ppm)	
	At zero time	After six months
Fe	30.490	21.4
Zn	2.642	1.20
Ni	2.264	0.90
Cu	5.733	3.40
Co	0.145	0.09
Cr	0.325	0.15
Mn	3.051	0.98
Pb	1.220	1.20
Cd	0.025	0.02

Composition of oily sludge biodegraded at HPCL refinery

At zero time (before initiation of bioremediation) composition of oily sludge biodegraded at HPCL refinery was estimated. Water content in sludge was recorded at 17%. Solvent extractable total petroleum hydrocarbon in sludge was 61%, organic matter was 15% and sediment/ash content was 11% (Table 8.3). TPH recovered from sludge was further fractionated into various fractions which showed 60% alkane fraction, 30% aromatic fraction, 3% NSO fraction and 7% asphaltene fraction (Table 8.3).

Table 8.3 Constituents of oily sludge biodegraded at HPCL Refinery

Constituents of oily sludge	Composition (%)
Water	13
Solvent extractable total petroleum hydrocarbon	61
Organic matter	15
Sediment/ash	11
<u>Constituents of TPH</u>	
Alkane fraction	60
Aromatic fraction	30
NSO fraction	3
Asphaltene fraction	7

In-situ Bioremediation of oily sludge

A pit contained approximately 350 metric tonnes of oily sludge was selected for bioremediation. In the month of May 2000, Oilzapper (200 kg) and nutrients (200 litres) was applied on oily sludge. At zero time the solvent extractable TPH in oily sludge was recorded 52.81%. Treatment of this sludge with Oilzapper showed that solvent extractable TPH (oil content) in sludge reduced from 52.81% to 23.59% in two months, from 52.81% to 8.33% in four months and from 52.81% to 6.07% in six months which revealed 55.33%, 84.2% and 88.5% biodegradation of oily sludge in two, four and six months respectively (Figure 8.2). Oily sledge in pit at HPCL refinery has been completely biodegraded reclaimed by application of oilzapper as shown in Figure 8.3.

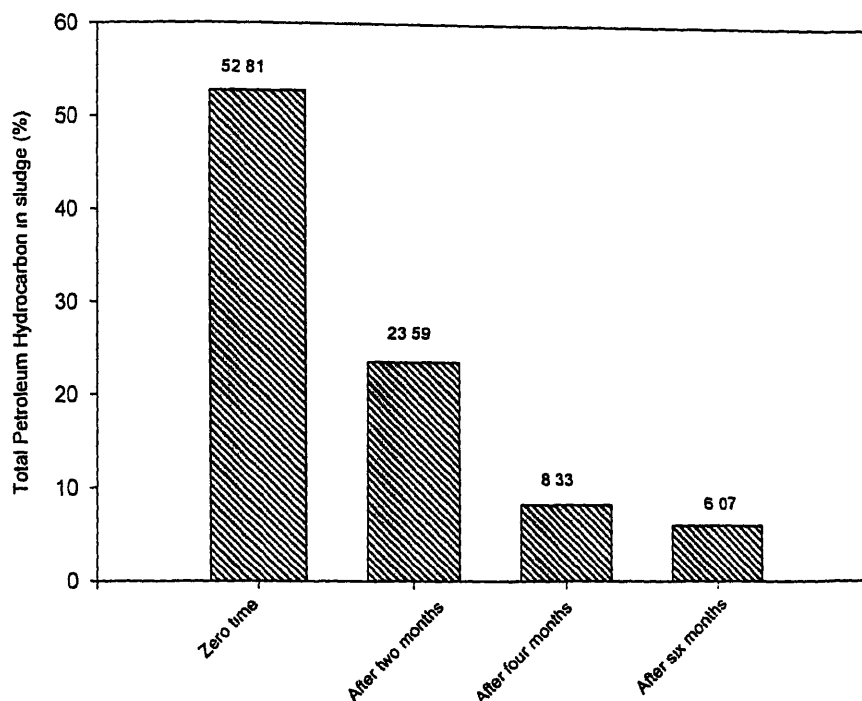


Figure 8.2 Total petroleum hydrocarbon in oily sludge during bioremediation of oily sludge

Biodegradation of various fractions of oily sludge

Biodegradation of various fractions of oily sludge was estimated. With application of Oilzapper and nutrients on oily sludge, 88.5% oily sludge (total petroleum hydrocarbon) was biodegraded in six months. Out of 88.5% biodegradation of oily sludge, biodegradation of alkane fraction was accounted for 55.4%, aromatic fraction 26.3% and NSO plus asphaltene fraction 6.8% (Table 8.4).

Table 8.4 Biodegradation of various fractions of oily sludge

Fractions	Biodegradation (%)
	Sludge treated with Oilzapper + Nutrients
Total petroleum hydrocarbon	88.5
Alkane	55.4
Aromatic	26.3
NSO + asphaltene	06.8

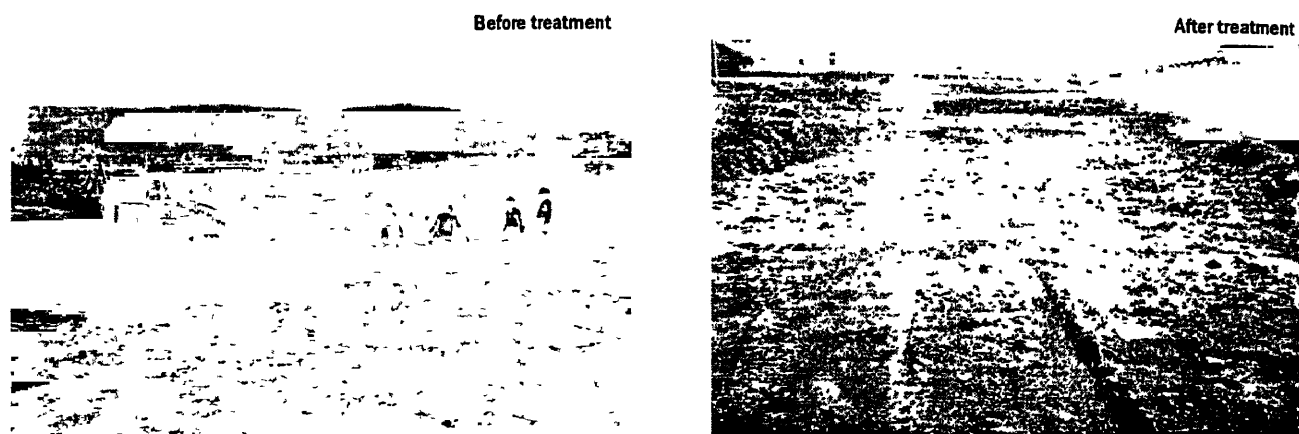
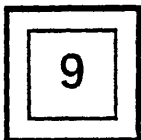


Figure 8.3 Biodegradation of oily sledge in sledge pit by oilzapper

Summary

- Bioremediation of oily sludge at HPCL Refinery, Visakhapatnam was initiated in the month of May 2000. A pit (approximately 30 m x 20 m) contained more than 350 metric tonnes of oily sludge was undertaken for bioremediation. In the month of May, July and September 2000, Oilzapper and nutrients were applied on oily sludge for biodegradation.
- At zero time (just after application of Oilzapper on sludge), oil content (TPH) in sludge was 52.81%. With application of Oilzapper and nutrients, the oil content in sludge reduced from 52.81% to 6.07% in six months which revealed 88.5% biodegradation.
- In Oilzapper treated sludge, a total 92.3% alkane fraction, 89.6% aromatic fraction and 62% NSO and asphaltene fractions of TPH were biodegraded in six months.
- Heavy metals concentration in soil at sludge treatment site reduced significantly in six months. The concentrations of heavy metals recorded in soil at bioremediation site were within the permissible limits and these concentrations were not having any adverse effect on soil micro-flora and fauna.



Conclusion

Crude oil and oily sludge degrading bacterial consortium was developed by assemble of five bacterial strain which could degrade alkane, aromatic, NSO (nitrogen, sulphur, oxygen containing compounds) and asphaltane fractions of crude oil and oily sludge.

Crude oil and oily sludge degrading efficiency of oilzapper was tested under laboratory and field conditions.

A feasibility study on bioremediation of crude oil and oily sludge contaminated site using oilzapper was conducted at Mathura refinery. The encouraging results of feasibility study prompted us to conduct multi-location field trials on bioremediation of crude oil and oily sludge contaminated sites using oilzapper.

Multi-location field trial on bioremediation of crude oil and oily sludge contaminated sites using oilzapper was conducted at Mathrua refinery, Barauni refinery, Digboi refinery, Guwahati refinery, HPCL refinery, Visakhapatnam, BPCL refinery, Mumbai and Oil India Ltd, Duliajan.

- With application of oilzapper at Mathura refinery 2.5 acre land contaminated with more than 1000 metric tonnes of oily sludge has been cleaned up.
- At Barauni refinery more than 5400 metric tonnes oily sludge has been treated by application of oilzapper.
- At Guwahati refinery more than 80 metric tonne oily sludge has been treated with application of oilzapper.
- At Digboi refinery more than 500 metric tonnes oily sludge has been treated with application of oilzapper.
- At Oil India Ltd, Duliajan more 500 metric tonnes oily sludge has been treated with application of oilzapper and a water pit contaminated with oil slick has been cleaned up using oilzapper.
- At BPCL refinery, Mumbai more than 300 metric tonnes oily sludge has been treated by oilzapper.
- At HPCL refinery, Visakhapatnam more than 350 metric tonne oily sludge has been treated by oilzapper.

Now bioremediation technology using oilzapper has been transferred for commercialization to Shriram Biotech Ltd, Hyderabad and Bharat Petroleum Corporation Limited, Mumbai for a technology fee of Rs.55 lacs and 5 lacs as royalty.

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